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[GANN, 48, 129~137; June, 1957]

CARCINOGENIC ACTION OF 4-NITROQUINOLINE-N-OXIDE

(Plates X-XII)

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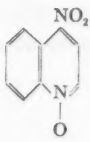
INTRODUCTION

According to the postulate of Haddow (1) the substances inhibiting cancer growth may be expected to be also carcinogenic, and *vice versa*. Although there is no strict parallelism between carcinostatic and carcinogenic actions of any given substance, this postulate is of interest in suggesting that a common factor or factors may be involved in these two apparently opposite biological processes.

Recently it was reported from this laboratory that certain quinone and quinoline derivatives possess a marked tumoricidal action *in vitro* and inhibiting action *in vivo* on ascites tumors (2, 3). The substances emerged as of special interest were alkylthio derivatives of 1, 4-naphthoquinone and 4-nitroquinoline-N-oxide and its derivatives, and, in view of Haddow's postulate mentioned above, we have undertaken to probe the possible carcinogenicity of some of these compounds. In the present paper we describe that part of our experiments which demonstrates that 4-nitroquinoline-N-oxide is a powerful carcinogen.

4-Nitroquinoline-N-oxide is one of the closely allied compounds synthesized for the first time by Ochiai and his collaborators (4, 5) by preparing N-oxide of quinoline by means of hydrogen peroxide and then by adding nitro radical.



The substance completely kills mouse sarcoma cells *in vitro* at the concentration of 0.002 percent, and greatly reduces their viability at 0.001 percent (3). In *in vivo* experiments using Ehrlich ascites carcinoma, it strikingly prolongs the average survival period in appropriate doses. It also inhibits the growth rate of subcutaneously transplanted Ehrlich carcinoma to a small but perceptible extent. The glycolysis-inhibiting action *in vitro* of the substance on cancer cells is demonstrable at the concentration of 4×10^{-4} M, although on this point it falls far short of certain other closely related derivatives (6). According to Okabayashi (7) the substance is also fungicidal as well as mutagenic to *Aspergillus niger*. Sakai and co-workers (8) demonstrated a very marked fungicidal action *in vitro* on *Trichophyton asteroides* and *Candida albicans*.

The carcinogenic action of 4-nitroquinoline-N-oxide has not hitherto been tested,

so far as we are aware. Previous reports on quinolines along this line are concerned with heterocyclic derivatives, such as β-anthraquinoline, 5, 6-benzoquinoline, and naphthoquinoline derivatives (Josephs (9), Shear (10)), none of them showing any sign of carcinogenicity. Quite recently, Umeda (11), in our laboratory, found 8-oxyquinoline to be non-carcinogenic.

Experimental Methods

The sample of 4-nitroquinoline-N-oxide used was kindly donated by Dr. Sumio Sakai, the Kaken Chemicals, Ltd., Tokyo, in whose laboratory it was prepared. The substance was yellow crystals, mp. 150–152°C, giving very pale yellow solution in benzene.

Animals used were normal males of a mixed strain mice, initialy weighing 16–18 g. Hair was pulled off over a small area of the interscapular region, and 0.25 percent solution of 4-nitroquinoline-N-oxide in benzene was applied on the depilated area of the skin three times a week by means of a small glass pipette. The amount of the solution was about 1/50 cc for each application, equivalent of 0.05 mg of the substance.

The treatment was discontinued when the macroscopic appearance of the tumor produced warranted the tentative diagnosis of malignancy.

Throughout the entire experimental period the mice were maintained on our usual laboratory diet of whole wheat grain supplemented with dried fish, with frequent supply of fresh carrots.

Usually mice were killed when they were found in weak physical state, although a few were inadvertently allowed to die. In all cases careful autopsy was performed, and tumors were examined histologically. In several cases, where there were sufficient amount of tumor tissues, their glycolytic activity *in vitro* was determined. Attempts to transplant the tumors were also made in a few cases, in spite of the very poor prospect of success due to the superficial nature of the growth with consequent infection.

Experimental Results

The early change observed in the treated area of the skin was the suppression of the re-growing of hair. In 60 to 80 days the skin area became more or less completely and permanently alopecic, and papillomatous growths began to appear in the area in about 100 days (Pl. X, Fig. 1). Many of these growths were solitary as they were first noticed, but other small nodular growths soon became apparent in their proximity. The solitary papillomas may break off and be replaced by new ones at the same site. Eventually they developed into hard dome-shaped growths with broad bases, and from that stage on they grew rapidly to form

large, irregularly roundish tumors with infiltrating basal layer, justifying the clinical diagnosis of malignant growth (Pl. X, Fig. 2). In most cases, the growths were accompanied by voluminous piling-up of hornified material, which produced irregular frill horns, usually several in a cluster (Pl. X, Fig. 3).

In the present experiment, started with 20 mice, 6 died within the first 100 days without showing any tumorous growth. The earliest malignant tumor was noticed on 120th day. There were 14 mice surviving at that time, and the rate of the development of papillomata and malignant tumors in these 14 mice is illustrated in Chart 1.

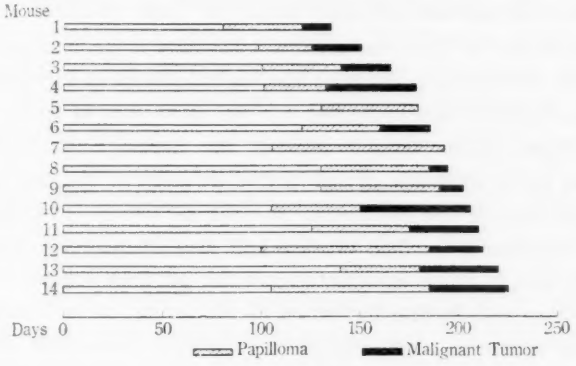


Chart 1. The rate of tumor production in 14 mice treated percutaneously with 0.25 percent solution in benzene of 4-nitroquinoline-N-oxide three times weekly.

As may be seen from the Chart, 5 of these 14 mice showed papillomata at the 100 day period. By the 150th day 5 mice (36 percent) bore malignant tumors, all the remaining mice showing papillomatous growths. Six mice survived 200 days, and in all of them tumors diagnosed as malignant were found.

In a general term it may be said that papillomata developed in all the mice during the period between 80th to 140th day, and malignant tumors between 120th to 200th day. At 150th day, 100 percent of the mice bore tumors, and in 36 percent the tumors were already malignant.

Histological Examination of the Tumors

The tumors produced in the above described experiment were generally characterized by the presence of multiple frill horns. Underneath these hornified growths, histological examination revealed squamous cell carcinoma of the type so familiar to every student of epidermal carcinogenesis. Irregular cords and masses of various sizes, composed of characteristic cancer cells, were seen growing invasively (Pl. XI, Fig. 4). Mitotic figures were frequent among cancer cells. In some areas there were formations of numerous pearls, while in other areas cystohemorrhagic changes were prominent. Some of the carcinomatous growths

were apparently associated with the wall of large keratinizing cysts.

The findings in the two mice (Mice 5 and 7), which did not show a large tumor at autopsy may be of interest. These two mice were killed in molibund condition on 179th and 192nd day, respectively. Both showed much piling up of hornified material over the treated area of the skin. These encrustations came off easily when attempt was made to remove them, revealing very irregularly hyperplastic layer of epidermis, which, upon histological examination, proved to contain many small nodules of the character suggestive of early squamous cell carcinoma (Pl. XI, Fig. 5). Many of the apparent papillomata which invariably preceded the large growths are probably of the similar histologic nature to these nodules.

Somewhat to our surprise, a few of the large tumors, especially those produced early in the above experiment, showed the undoubted histological picture of sarcoma (Mice, 1, 2, 4, and 9). These tumors were composed for the most part of irregularly interlacing bundles of long spindle shaped cells of the fibrosarcoma type (Pl. XI, Fig. 6). There was much evidence of the active invasion of the tumor tissue into the muscular layer and of the infiltration into the fat tissue. In these tumors there were varying degrees of surface erosion and also of necrosis and hemorrhage. Of special interest in these sarcomata were the pictures suggestive of the participation of squamous cells in the formation of the tumor, as indicated by the presence within the tumor of what appears to be a small squamous cell carcinoma (Pl. XII, Fig. 7). The behavior of the hair follicles also rendered difficult the exclusion of the follicular cells from the histogenetic consideration. The exact character of the tumor cells was difficult to determine in many places. It seems probable that although both epithelium as well as connective tissue of the dermis undergo malignant change under the influence of 4-nitroquinoline-N-oxide, the reaction of the epithelial cells, though taking place earlier, does not progress as rapidly as that of the connective tissue cells. These latter, once becoming malignant, could grow so much more rapidly that the slow-growing epithelial nodules might become more or less completely obliterated.

From the production of pure carcinoma on one hand and of sarcoma on the other, the occurrence of a mixed tumor may well be presupposed. It is probable, however, that such a mixed tumor, even if produced, may eventually become entirely a sarcoma on account of the greater proliferative activity on the part of the sarcomatous element. We have not found a single case which can be frankly diagnosed as carcino-sarcoma in our present series.

It is diffiicult to say why one and the same carcinogen, similarly applied, should produce carcinoma in some individuals and sarcoma in others. Difference in the degrees of incidental surface erosion may have something to do with the phenomena.

To recapitulate, the histological diagnosis of all the tumors was squamous cell carcinoma, with the following exceptions: sarcoma in Mice 1, 2, 4, and 9, papilloma or early carcinoma in Mouse 7, and papilloma in Mouse 5.

Metastases: Probably due to the relatively short period of survival of the mice after the development of malignant tumors, metastasis was not found as frequently as might be expected.

Metastasis to one of the submaxillary lymph nodes was found in Mouse 3. In this case almost the entire node was replaced by carcinomatous tissue which showed extensive central necrosis with keratinization (Pl. XII, Fig. 8), the histological characters corresponding to those of the primary tumor.

A small sarcomatous metastasis was detected in the case of Mouse 2. It occurred periarterially close to but outside one of the cervical lymph nodes (Pl. XII, Fig. 9).

No lung metastasis was found in any of the mice.

Liver Changes: At autopsy it was noted that while most of the internal organs showed no grossly apparent changes, the liver usually was not only much enlarged but was elastically hard to the touch and pale in color. Histologically these livers showed the picture of what appeared to be generalized amyloid infiltration to varying extents.

Some of these livers were examined as to their catalase activities, and they all showed marked decrease in the activity. Unfortunately, from the point of view of toxohormone effect, the significance of the reduced catalase activity was lost in these cases, because of the above mentioned histological changes, which greatly complicated the matter.

Glycolytic Activity of Tumor Tissues

By means of the classical Warburg technique the glycolytic activity *in vitro* was examined of the tumors produced in the above experiment whenever the size of the tumor permitted sparing sufficient amount of tissue for this purpose.

To a Warburg vessel was placed 3 ml of Krebs-Ringer-bicarbonate buffer solution containing glucose in the concentration of 200 mg/dl, and about 60 mg of slices of fresh tumor tissue. A mixture of 95 percent nitrogen and 5 percent carbon dioxide was used for gas phase. After the temperature equilibrium time of 10 minutes, the carbon dioxide produced by lactic acid formation in bicarbonate buffer was measured at 10 minutes interval for 1 hour, according to the established standard method. In this way we obtained the amount in μl of CO_2 per mg of fresh tissue per hour for five tumors, as tabulated below (Table 1). Data on respiration are also included insofar as determined.

For comparison the CO_2 values obtained by the identical method for various transplantable tumors may be cited as follows: 2.80–3.27 μl for Umeda's rhodamine

Table 1. Glycolytic Activity of Tumor Tissues

Tumor	Glycolysis CO_2 μl/mg/hr	O_2 uptake μl/mg/hr
1. (Mouse 2) Sarcoma	0.78	—
2. (Mouse 4) Sarcoma	2.21	0.45
3. (Mouse 3) Carcinoma	1.51	—
4. (Mouse 10) Carcinoma	1.52	0.40
5. (Mouse 13) Carcinoma	0.80	0.55

sarcoma (rat), 3.20μl for sarcoma 180 (mouse), 1.41μl for NF sarcoma (mouse). The quinoline-induced tumors may thus be said to include those with relatively low glycolytic activity, though generally conforming to the malignant tumors.

The possibility of a trace of 4-nitroquinoline-N-oxide, which is known to inhibit glycolysis, contained in the tumor tissue slices used is regarded as very remote, especially since the application of carcinogen had been discontinued for at least a few days before the glycolysis tests were made. It may be that the tumors produced by a glycolysis-inhibiting carcinogen are not endowed with very high glycolytic activity, but it must be remembered that high glycolysis is not always a characteristic of all the malignant tissues. It is well known that many spontaneous mammary cancers in mice show relatively low glycolysis (15).

As may be expected, the tissue respiration, as indicated by O_2 uptake, was low in all the tumors examined in the above experiment.

Transplantation Experiments

Because the tumors produced in the present experiments were of the skin, with varying degrees of surface erosion and consequent infection, attempts to transplant these tumors may be expected to be attended with considerable difficulties. With the majority of the tumors the prospect of successful transplantation seemed so poor that we did not even attempt it. However, we succeeded in transplanting one tumor out of three which we did try.

This tumor was that from Mouse 2, a sarcoma. It was transplanted subcutaneously into 5 normal mice by the usual trocar method. The grafts took in one mouse, in which the tumor reached the size of a thumb-tip in three weeks. Further transplantations were carried out starting with this tumor, resulting in the establishment of a transplantable strain which attained the 6th generation at the time of writing (NF sarcoma No. 2). The rate of positive transplantation rose rapidly, and 100 percent transplantability has been maintained in practically all the generations since the third.

Histologically, the strain as it stands today is a pure fibrosarcoma.

Discussion

The carcinogenic action of 4-nitroquinoline-N-oxide demonstrated in the above described experiment is of special interest for several reasons. In the first place it introduces a bicyclic compound into the families of powerful carcinogens, which have hitherto included certain tetra- or pentacyclic hydrocarbons, aminoazo dyes and aromatic amines. The fact that the carcinogenic potency of 4-nitroquinoline-N-oxide is of the same order of magnitude at least as that of 1, 2, 5, 6-dibenzanthracene, if not that of 20-methylcholanthrene, suggests the possibility that a carcinogen of even greater power may be found among its various derivatives. We strongly suspect that the nitro radical may be importantly related to the carcinogenicity of this class of compounds, and the elucidation of the relation between the chemical structure and carcinogenic action calls for an extensive comparative assay of many quinoline derivatives.

The histological character of the tumors produced may be mentioned as another point of interest in the present experimental result. The tumors induced by the skin painting using polycyclic hydrocarbons were almost always squamous cell carcinomas, while our present series of quinoline-tumors included a fair proportion of fibrosarcoma. This fact may suggest a certain difference in the tissue affinity of 4-nitroquinoline-N-oxide from that of the carcinogens of the polycyclic hydrocarbon group, the point which requires further investigation. It may be recalled in this connection, however, that the skin tumors produced by exposure to ultraviolet light in rat and mouse start in the dermis and hence are sarcomata, while in man such tumors are usually epitheliomata, obviously depending on the degrees of the penetration of the light of the active wave length (2600 to 3400 A°) (12, 13).

From a biochemical point of view it may be significant that Fukuoka, Sugimura and Sakai (6) found that 4-nitroquinoline-N-oxide is among those quinoline derivatives which possess marked inhibiting action on the glycolytic function but not on respiratory activity of tumor cells. We are well aware that not all the inhibitors of glycolysis are carcinogenic, and if one follows Warburg's classical theory (14), one may expect an inhibitor of cell respiration but not any inhibitor of glycolysis to be cancer producing.

This discussion takes us back to the starting point of our present investigation, namely, the paradoxical postulate of Haddow (1) that chemicals which cause cancer may also cure it, and *vice versa*. Any chemotherapeutic effect must mean injury of some fundamental sort to cancer cells. What is the mode of cancericidal action of quinoline derivatives? The work of Fukuoka *et al.* (6) implicates the inhibition of glycolysis in this mechanism, but it does not exclude other possibili-

ties. It may be recalled that Ochiai (16) early pointed out that the nitro radical at the position 4 of quinoline-N-oxide is very active in its electronic property and can be easily substituted by nucleophilic substances. Okabayashi (7) attributed the fungicidal action of 4-nitroquinoline-N-oxide to the substitution reaction of the nitro radical with SH group. There seems to exist a fertile field for investigation in the biological and biochemical activities of the 4-nitroquinoline-N-oxide derivatives, which are cancericidal as well as very strongly carcinogenic.

Summary

4-Nitroquinoline-N-oxide was demonstrated to possess a very powerful carcinogenic action. Applied to the skin of normal mice three times a week this substance in 0.25 percent benzene solution produced papillomata in all the animals during the period between 80th to 140th day, and malignant tumors between 120th to 200th day. At 150th day, 100 percent of the mice bore tumors and in 36 percent the tumors were already malignant. The malignant tumors produced were histologically squamous cell carcinoma or fibrosarcoma. Metastases were found in a few cases. These tumors were found to show the glycolytic activity *in vitro* consistent with their malignant nature. One of the tumors was successfully transplanted to normal mice.

4-Nitroquinoline-N-oxide is the first bicyclic compound to be shown to have the carcinogenic activity of the magnitude approaching that of the most potent polycyclic hydrocarbons.

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Explanation of Figures

Plate X

Fig. 1. A papillomatous growth. Mouse 10 (120 days).

Fig. 2. A large roundish tumor. (This tumor proved to be a sarcoma). Mouse 2 (145 days).

Fig. 3. A large tumor with confluent frill horns. (This tumor proved to be a typical squamous cell carcinoma). Mouse 3 (160 days).

Plate XI

Fig. 4. An example of the usual type of malignant tumors produced: squamous cell carcinoma. Mouse 3 (165 days).

Fig. 5. A part of irregular epithelial hyperplasia or early squamous cell carcinoma, not yet producing a large tumor. Mouse 7 (192 days).

Fig. 6. A typical fibrosarcoma. Mouse 1 (134 days).

Plate XII

Fig. 7. A sarcoma containing an epithelial nodule suggestive of early squamous cell carcinoma. Mouse 2 (150 days).

Fig. 8. A lymph node metastasis of squamous cell carcinoma. Mouse 3 (150 days).

Fig. 9. A periarterial sarcomatous metastasis in the neck region. Mouse 2 (150 days).

要　旨

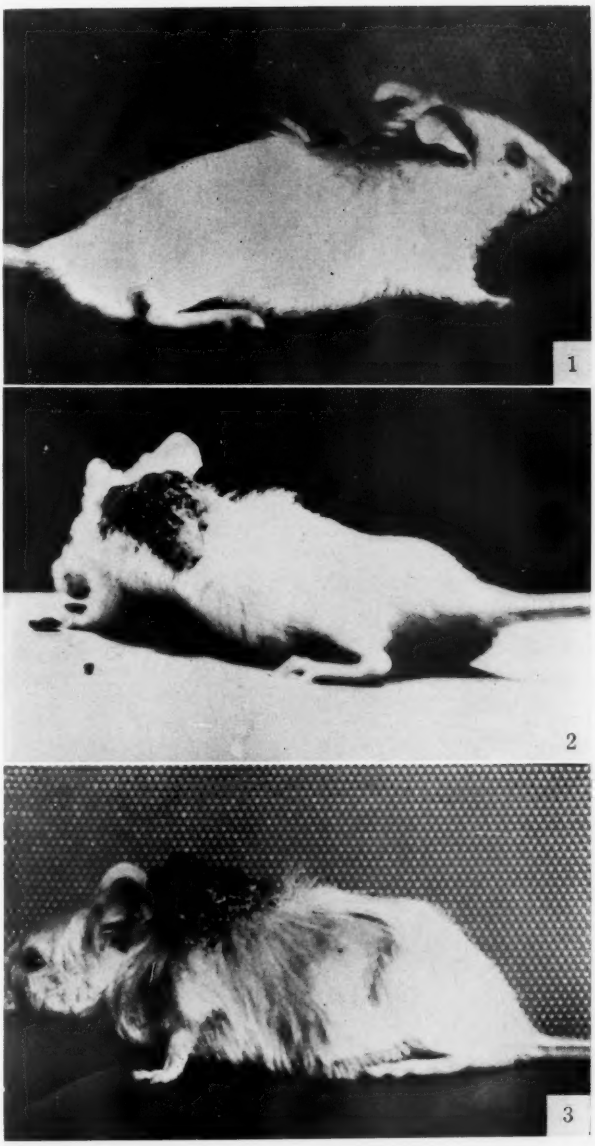
4-Nitroquinoline-N-oxide の癌原性

中原和郎，福岡文子，杉村　隆
(癌 研 究 所)

現在続行中のキノリン誘導体の癌原性に関するわれわれの研究のうち，4-nitroquinoline-N-oxide が極めて強力な発癌性を有するという事実を報告した。マウスの皮膚に 0.25% ベンゼン溶液として1週3回塗布すると 80-140 日で全例にパピロマを発生し，120-200 日で悪性化する。できた腫瘍は普通の扁平上皮癌の外に相当数の線維肉腫を含んでいる。何れも悪性腫瘍に特徴的な解糖作用を示す。転移は2例に証明された。移植は表在性腫瘍であるため非常に困難であるが，試みた3例中1例において成功した。

4-Nitroquinoline-N-oxide の発癌力は最強力な多核炭化水素のそれに接近している。キノリンの如き比較的簡単な二核化合物中にこのような強力な癌原性物質があることは予期されなかったところである。

(文部省科学研究費による)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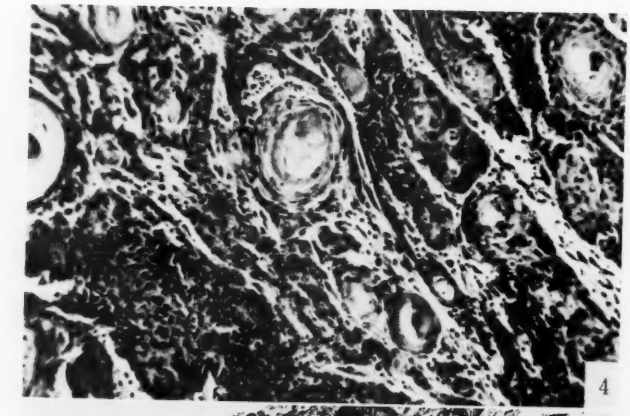
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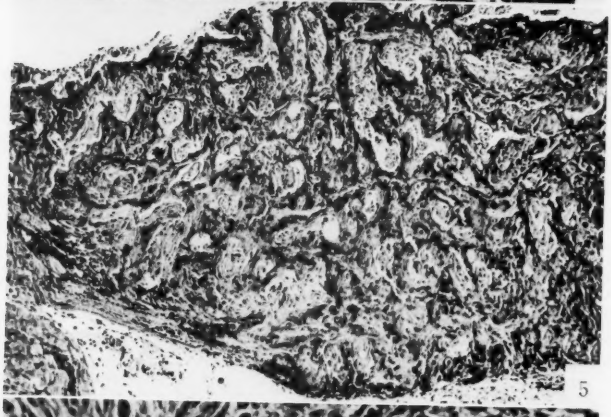
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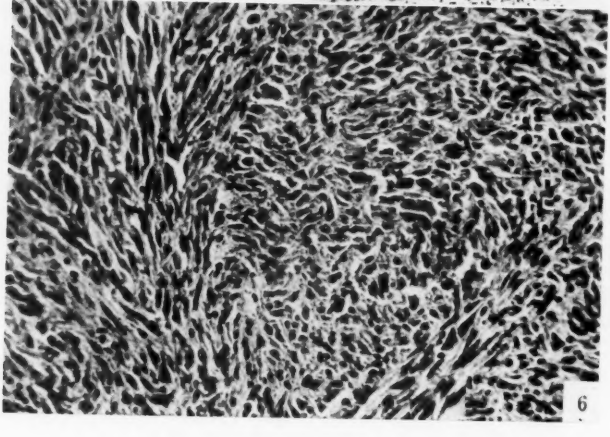
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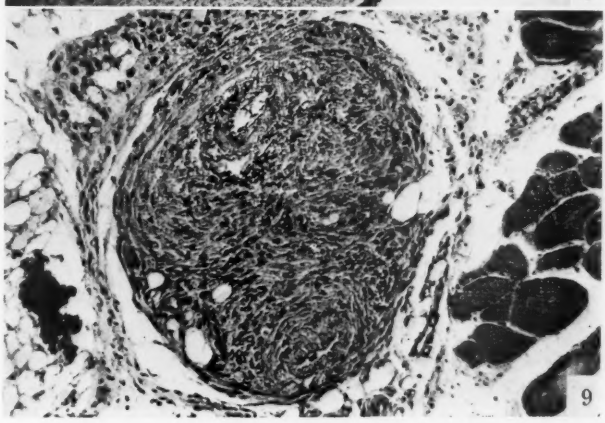
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[GANN, 48, 139~144; June, 1957]

PRODUCTION OF RAT SARCOMA BY INJECTIONS OF PROPYLENE GLYCOL SOLUTION OF p-QUINONE

(Plate XIII)

MASAO UMEDA

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In 1955 (1), I reported on the experimental production of rat sarcoma by injections of m-toluylenediamine. In that experiment, I used propylene glycol as solvent for m-toluylenediamine, instead of using water, with the surprising result that sarcoma developed in 100% of rats, in spite of the fact that m-toluylenediamine has hither to been regarded as non-carcinogenic. Here the first question that arises is whether or not propylene glycol is in itself carcinogenic. But this question is answered in negative by the control experiment using propylene glycol alone, as may be evident from Experiment II described in this paper.

In the present experiment, the carcinogenic effect of p-quinone was examined by repeated subcutaneous injections of its solution in propylene glycol. Carcinogenic activity of p-quinone has been tested in the past by Takizawa (2), Sugishita (3), Kishizawa (4), etc., by skin painting or inhalation experiments which showed that this substance produces skin tumors and lung tumors in a small percent of the mice after a prolonged treatment. Production of sarcoma by subcutaneous injections has never been reported.

EXPERIMENTS

Experiment I. In this experiment, the effect of p-quinone (1, 4-benzoquinone; $C_6H_4O_2$) injections was examined. This substance is said to be used as oxidizing agent, and also in photography.

Animals used were 15 rats of Wistar strain and 9 of hybrid strain weighing around 200 g.

The preparation of p-quinone used in the experiment was the highest purity product of the Tokyo Kasei Industry Co., Ltd., Tokyo, and propylene glycol ($CH_3CHOH \cdot CH_2OH$) was a product of the Junsei Chemical Co., Ltd., Tokyo, also of the superior quality.

p-Quinone was dissolved in propylene glycol at the concentration of 1 gdl, and was injected subcutaneously on the back of the rats in 0.5 cc amounts. Injections were repeated once every week, and were delivered into as nearly the same site as possible. The concentration was reduced to 0.2 gdl after 53 days, and was

increased to 0.4 gdl after 173 days. p-Quinone solution was prepared freshly every time, and sterilized by heating before injection.

Results: Of the 24 rats, with which the experiment was started, 17 rats survived the injection period of 394 days, receiving 32 injections (p-quinone: 81 mg, propylene glycol: 16.5 cc) during the period. Sarcoma developed in the 2 rats (Table 1).

Table 1. Experiment 1

Rat No.	Sex	Exp. days	Total amount injected		Inject. No.	Body Weight (Final)	Tumor size (cm)	Histological diagnosis
			p-Quinon	Propylene Glycol				
1	m	366	81	16.5	32	——		
2	m	410	83	17.0	33	200		
3	m	415	83	17.0	33	200		
4	m	423	81	16.5	32	275	5.2×5.4×3.6	Fibrosarcoma
5	/	439	87	18.0	35	240		
6	m	474	95	20.0	37	218		
7	f	481	85	17.5	34	233	3.9×3.5× 2.2 +2.7×2.1×1.2	Fibrosarcoma
8	/	494	97	20.5	39	150		
9	f	525	105	22.5	43	187		
10	m	529	105	22.5	43	268		
11	f	541	109	23.5	45	176		
12	m	566	111	24.0	46	300		
13	m	567	111	24.0	46	296		
14	m	568	111	24.0	46	266		
15	f	573	111	24.0	46	——		
16	m	582	115	25.0	48	184		
17	f	585	115	25.0	48	166		

Individual records of the 2 rats which developed sarcoma are as follows:

No. 4. A nodule measuring 3.7×3.0×2.0 cm was palpated on the 394th day. The animal died on the 423rd day with the tumor measuring 5.2×5.4×3.6 cm.

No. 7. The tumor was found as a hard nodule of the size of small finger tip on the 417th day, which grew to the size of thumb-tip by the 429th day. The tumor reached the size of 3.9×3.5×2.2 (and 2.7×2.1×1.2) cm on the 481st day. The animal was killed in very weakened condition on that day. A macroscopic metastasis was found in the left axillary lymph node.

(No. 12 in the table, dying on the 566th day, had a cysticercus sarcoma (spindle cell sarcoma) of the liver.)

Findings at autopsy: These tumors were found in the subcutaneous tissue at the site of the injections, the shape showing some irregularities. They were elastically hard, cut surface evenly pearly white with hardly recognizable necrotic

or hemorrhagic areas. No significant change was noted in internal organs.

Transplantation: Attempts to transplant the tumor to normal rats were made in the both cases. The transplantation from No. 7 tumor took in 70% of the animals in the first generation, 55% in the second, 50% in the third, 66% in the fourth, 66% in the fifth, 38% in the sixth, 70% in the seventh, etc. In the case of No. 4, tumor tissue for transplantation was taken from animals that has been dead, and probably for this reason transplantation did not succeed. These transplanted rats died of tumors in about one month on an average after transplantation. Microscopically, the transplanted tumors were classed as fibrosarcoma identical with the original tumor.

Histology of the tumors: The tumors of both No. 4 and No. 7 rats were diagnosed as fibrosarcoma, being composed chiefly of spindle cells. Mitoses were numerous in most part of the tumors, and giant cells and polymorphic cells were found only in limited areas. The lymph node metastasis found in No. 7 rat, coincided with the primary tumor in every histological feature.

Failure of Sarcoma Production by Injections of Propylene Glycol

Experiment II: Propylene glycol was tested in this experiment.

Experiment under the usual conditions was started with 18 normal rats of a mixed strain from the Saitama Prefecture, all weighing around 200 g.

The preparation of propylene glycol ($CH_3CHOH \cdot CH_2OH$) used in the experiment was the same sample used as solvent for p-quinone in Experiment I.

Injections were made subcutaneously on the back of the rats at as nearly the same site as possible every time. The injection of 1 cc of propylene glycol was

Table 2. Experiment II

Rat No.	Sex	Exp. days	Propylene Glycol		Final Body Weight (g)
			Total (cc)	Inject. No.	
1	m	362	47	47	190
2	m	363	47	47	295
3	m	390	51	51	295
4	m	403	52	52	270
5	m	482	60.5	63	410
6	m	554	65.5	72	355
7	m	556	65.5	72	278
8	m	557	65.5	72	340
9	m	559	65.5	72	322
10	m	567	66.5	74	220
11	m	614	69.5	80	233

repeated once every week as a rule and after about 15 months, the amount was reduced to 0.5cc.

11 rats survived 300 days or more, one of the highest longevity surviving 614 days (Table 2). No tumor was produced at the site of injection in any of the rats.

The local tissue at the site of the injections showed chiefly hyaline fibrosis. No significant change was noted in internal organs at autopsy. However, in the course of the experiment, one of the rats, dying on the 403rd day, showed adenoma of the hypophysis (small pea size), probably of spontaneous origin.

Discussion

Some years ago Professor Y. Asahina suggested that quinones produced as the terminal metabolites of carcinogenic azo dyes as well as of hydrocarbons might be the true active substances in the effect of these well known carcinogens. Based on this suggestion, Takizawa (2), then of this laboratory, carried out animal experiments using several kinds of quinone, and reported that some of them are indeed carcinogenic, though disappointingly weakly. Kuhn and Beinert (5) later expressed a similar opinion, but Takizawa's results must be regarded as far too inadequate to support the hypothesis.

Also, Takizawa (6) supposed that chinhydron ($O=\langle\rangle=O\cdot HO\text{-}C_6H_4\text{-}OH$) into which quinone changes in light may be carcinogenic. This point has not been settled.

However, in the above described experiment, I succeeded in producing sarcoma in rats by injections of p-quinone at any rate, and in Experiment II, it was proved that propylene glycol, used as solvent, has no carcinogenic activity. I think that propylene glycol is the promoting factor as in the case of my previous experiments with m-toluylenediamine. It seems possible that propylene glycol may facilitate the penetration of p-quinone into the cell, but further studies are needed to discuss this point. Some new implications in the mechanism of the promoting action in carcinogenic processes will be discussed in future.

Summary

1. Sarcoma was produced at the site by repeatedly injecting 0.2—1.0 gdl solution in propylene glycol of p-quinone subcutaneously into normal rats.

2. The use of propylene glycol alone resulted in no tumors during a 88 week course of the injections.

3. No notable change in internal organs was found in the rats in these experiments.

Acknowledgements

I take pleasure in acknowledging my indebtedness to Dr. Waro Nakahara and Dr. Makoto Tanaka for their help and encouragement in the course of this work.

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Explanation of Figures (Plate XIII)

Fig. 1. Fibrosarcoma, arising from the site of the administration of p-quinone on the 394th day.

Fig. 2. Histological picture of the above tumor.

Fig. 3. Histology of the lymph node metastasis in rat No. 7.

要　旨

p-Quinone によるラッテ肉腫の実験的成生

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p-Quinone による発癌実験は滝沢などによつて，塗布あるいは吸入実験が行われ，何れも癌の発生を認めている。しかしいまだ *p*-Quinone による肉腫の発生は認めていない。私はラッテを用い *p*-Quinone の propylene glycol 溶液を1週間1回宛，ラッテの背部皮下に注射し，394 日以上生存した17匹のうちの2匹に肉腫の発生を認めた (*p*-Quinone: 81～85 mg, Propylene Glycol: 16.5～17.5 cc). Propylene glycol のみの注射実験においては1例も肉腫の発生を認めなかつた。

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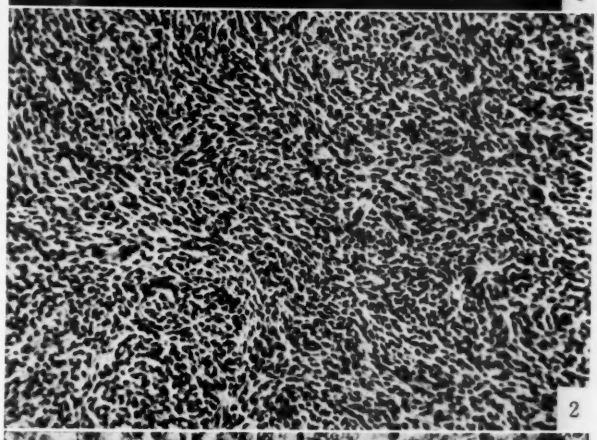
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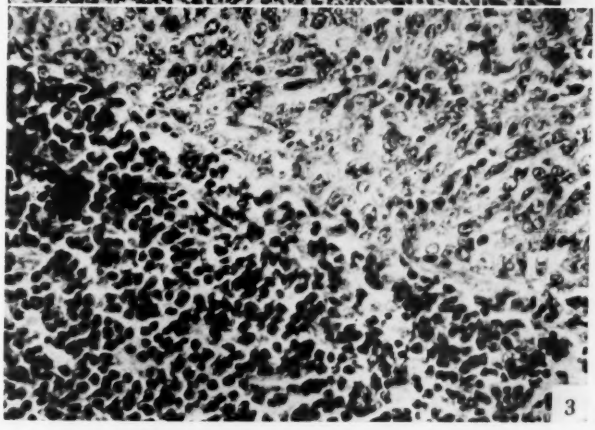
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[GANN, 48, 145~158; June, 1957]

INHIBITORY EFFECT OF P-HYDROXYPROPIOPHENONE (PHP) UPON EXPERIMENTAL INDUCTION OF HEPATOMA IN RATS FED WITH BUTTER-YELLOW (DAB)

(With Plates XIV–XVI)

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The successful induction of hepatoma in rats with the azo-dyes (Sasaki and Yoshida (1935), Kinoshita (1937)), has been followed by many important contributions to the knowledge of biology, biochemistry and pathology of cancer, and one of such contributions has been the discovery of substances acting as inhibitors in the azo-dye carcinogenesis.

Nakahara et al. in 1939 first reported high inhibitory potencies of the dried powder of beef liver, in protecting the rat's liver from DAB carcinogenesis. Their results have since been confirmed by many investigators, although the identity of the active substance involved and the mechanism of its action remain yet to be clarified. A number of pure organic chemicals have been proposed as inhibitors in the azo-dye carcinogenesis, and some of them may act as competitors in forming protein bound dye stuff as in the case of methylcholanthrene (Miller and Miller, 1954) and trypan blue (Kawai et al., 1952). On the other hand, importance of the role of pituitary hormones in experimental carcinogenesis has been emphasized recently. A large scale investigation in this concern had been lacking until Griffin et al. (1954) presented a series of data testifying that hypophysectomy in rats induced remarkable suppression of induction rate of DAB hepatoma, as well as other forms of experimental tumorigenesis. They found in addition that the inhibition could be overcome with growth hormone and ACTH.

While Griffin et al. applied surgical hypophysectomy, some investigators postulated that p-hydroxypropiophenone might exert an action of chemical hypophysectomy, so to speak, when given to animals per orally.

p-Hydroxypropiophenone (PHP) was first introduced by Dodd, (1938) as a new estrogenic substance. In chemical structure, PHP represents the exact one-half of a stilbesterol molecule. A rather peculiar inhibitory effect of this chemical upon secretion of both gonadotropin and TSH of the anterior pituitary gland was first reported by Perrault et al. (1949) and Lacassagne et al. (1950), although findings and opinions of various other investigators concerning the action and effects of the substance are diverse and appear never to have come to a definite agreement.

The present study was planned in the hope of reinvestigating the effect of surgical hypophysectomy and comparing with it the alleged chemical hypophysectomy in the course of the DAB carcinogenesis in rats. Robertson, Griffin and Richardson (1954) already reported an experiment almost identical in purpose and in arrangement, but they followed the hepatic changes in a much smaller group of animals and for a much shorter duration of time than seem critical to the experiment of this type. Intake of the carcinogen was not calculated as exactly as could have been desired. As it turned out, however, our results and conclusion reached are quite similar to theirs, and we agree also in discrediting the effect of PHP through the pituitary. The present report thus provides a confirmation and a support of their work on the basis of a larger and more exact program, complete with more detailed histologic examination.

Material and Experimental Method

Exclusively the male hybrid (Saitama line) rats were used. Their initial body weight ranged between 110 and 180 g. Five rats were kept in one cage.

The basic diet was prepared as follows:—

Crushed half-polished rice grain	5 kg
DAB* (3.0 g in 100 ml olive oil)	3 g (0.06%)
Casein**	25 g
Flake of dried bonito	50 g

In the first and second weeks of the experiments low DAB diets (0.02% DAB in the first week, and 0.04% DAB in the next) were given.

The diet was given every day at the rate of 10 g per capita and the remnants of the diet were weighed every day. Thus the diet and chemicals consumed were calculated on per cage basis.

The animals were divided in three groups: Group A received the basic diet only. Group B was fed with the basic diet plus PHP.*** (10 g PHP were dissolved in 100 ml of ether-alcohol a. a., and added to 1 kg of the basic diet, then the ether-alcohol was evaporated at room temperature to get a fairly homogeneous mixture.) The third group, Group E-F, consisted of rats hypophysectomized previously. Hypophysectomy was performed by the technique described by Tanaka, a modification of Koyama's external auditory canal method. Many animals died postoperatively. Rats survived one week were started upon feeding with the basic diet.

The experiments were terminated at the 160th day after beginning the feeding with the carcinogenic basic diet. All the the rats surviving at this period were

* Wako Pure Chemical, Ltd. ** Kokusan Chemical Work, Ltd. *** Koowa Chemical Co., Ltd.

sacrificed and following organs were weighed and examined histologically in details: the liver, spleen, kidneys, adrenals, testes, hypophysis, thymus and thyroid.

Result

The rate of incidence of hepatoma and mortality among the entire experimental series are shown in Table 1.

In Group A (DAB only group), in which the rats were fed exclusively with the basic diet containing DAB, hepatoma occurred in 54.5% of the animals which survived 160 days, while severe liver cirrhosis was seen in 100%.

The animals in Group B fed with the basic diet containning DAB and PHP simultaneously, developed, in a very sharp contrast to above, no hepatic tumor, i.e. hepatoma incidence was 0% in this series. Seventeen of 20 animals survived 160 days. The mortality rates in Groups A and B were 63.3% and 15%, respectively. Remarkably, none of the 17 rats in Group B showed cirrhotic lesion of the liver.

In Group E-F, the rats were hypophysectomized prior to the azo-dye feeding. 52.4% of the animals died prematurely. Among the 10 rats survived, 30% developed hepatomas and all showed cirrhosis of various grades of severity. All rats which were completely hypophysectomized, as it was verified by necropsy, died in the very early phase of the feeding experiment. Ten rats which survived 160 days showed either almost intact hypophyses or status of partial hypophysectomy. Three of the latter group of rats in which the pituitaries weighed 2 and 3 mg at necropsy, revealed only very slight hepatic cirrhosis and no hepatoma.

Feeding amount of the diet of the 10 rats of Group E-F was no less than in Groups A and B. Average amounts of DAB fed during the experiment were 523.02-577.80 mg, 572.60-586.56 mg and 527.72-577.88 mg in Groups A, B and E-F, respec-

Table 1. Hepatoma Incidence and Mortality in the Three Experimental Groups.

Experimental Groups	Number of rats		Experimental days	Findings of the liver				Comments
	Initial	Final		Normal	Cirrhosis	Adenoma	Hepatoma	
(A) DAB	30	11	160	0	11	7	6 (54.5%)	
(B) DAB+PHP	20	17	160	17	0	0	0 (0%)	Complete Inhibition
(E-F) DAB + Hypophysectomy	21	10	160	0	10	10	3 (30%)	Inhibition paralleling the grade of hypophysectomy

tively. Slight but clear advantage of the Group B in average azo dye intake can be seen. Since the caloric intake should go parallel with the azo dye consumed, the animals of Group B apparently had taken more food in average than the other groups.

Findings on the liver:

Group A: The weight of the livers ranged between 8.5 g and 18.6 g. Six showed irregularly nodular masses of a finger-tip to thumb-tip size, which proved to be all typical trabecular hepatomas on histologic examination (Fig. 9). Conspicuous atypism and irregularity of the nuclei, deep basophilia of the cytoplasm and frequent mitotic figures were noticed. Invasive character of the proliferating masses was also demonstrable. Necrosis occurred in the nodules often. Seven had nodular hyperlasia of the hepatic cells consistent with hepatocellular adenomas of the previous authors. In a few cases, cholangiocysts were observed. All the 11 rats, irrespective of the presence or absence of the nodular lesions above, showed advanced stages of cirrhosis associated with marked cholangiofibrosis (Fig. 8). Groups of the small round cells and deposits of yellow pigment granules in the stellate cells were frequently observed in the acini.

Group B: The weight of the livers ranged in this group between 5.5 g and 11.5 g. One had the liver weighing 23.0 g. However, this was due to the existence of multiple cysticercus cysts. All the other livers had quite smooth and glistening

Table 2. Gross findings of the organs (Group A—DBA only)

Rat No.	Body Weight (g)		Amount of DAB (mg) fed (average)	Liver		Organ Weight						
						Visceral			Interal Secretory			
	Initial	Final		Tumor	Cirrhosis	Liver (g)	Spleen (g)	Kidneys (g)	Hypophysis (mg)	Thymus (mg)	Adrenals (mg)	Testes (g)
1	118	225	523.02	+	+	15.0	0.9	2.0	8	250	60	2.8
2	128	240		±	+	11.8	0.75	1.8	7	195	40	1.8
3	120	205		±	##	16.5	0.4	1.4	6	265	38	3.4
4	140	180	545.10	+	+	8.5	1.2	1.6	6	245	28	2.2
5	145	215		#	#	17.5	1.6	2.0	7	259	26	3.2
6	130	217		#	#	18.6	1.5	2.1	5	247	21	3.1
7	140	195	577.80	#	#	11.5	1.7	1.6	4	306	23	2.9
8	126	210		+	+	10.2	1.4	1.8	6	383	20	3.6
9	140	240		±	#	15.5	2.7	2.3	7	241	31	4.2
10	160	250	547.32	±	##	13.8	0.7	1.6	7	235	30	3.9
11	148	225		±	#	12.5	0.8	1.7	9	255	44	2.7

Table 3. Gross findings of the organs (Group B—DAB+PHP)

Rat No.	Body Weight (g)		Amount of DAB (mg) fed (average)	Amount of PHP (g) fed (average)	Liver		Organ Weight						
							Visceral			Internal Secretory			
	Initial	Final			Tumor	Cirrhosis	Liver (g)	Spleen (g)	Kidneys (g)	Hypopnysis (mg)	Thymus (mg)	Adrenals (mg)	Testes (g)
1	118	225	586.56	10.36	—	—	11.5	0.6	1.6	6	142	32	2.9
2	128	186			—	—	7.9	0.6	2.7	4	143	21	2.7
3	102	145			—	—	7.5	0.55	1.0	3	154	21	2.3
4	108	150			—	—	6.3	0.45	1.2	4	96	25	2.0
5	128	225			—	—	9.7	1.0	1.4	4	220	30	2.8
6	134	230	572.60	10.08	—	—	8.8	1.1	1.4	7	134	32	2.5
7	138	205			—	—	8.1	1.0	1.3	6	280	28	3.1
8	126	200			—	—	8.1	1.2	1.2	7	280	29	3.3
9	132	245			—	—	9.1	1.3	1.6	6	208	25	3.3
10	132	270	582.64	10.24	—	—	8.1	0.75	1.5	5	210	24	3.0
11	142	240			—	—	7.8	0.6	1.6	5	245	30	2.9
12	134	205			—	—	7.4	0.4	1.4	4	257	26	3.0
13	134	225			—	—	5.8	0.4	1.2	4	252	18	2.4
14	160	185	579.04	10.18	—	—	5.0	0.5	1.2	3	201	32	2.4
15	144	270			—	—	8.4	0.75	1.5	4	314	31	4.1
16	142	225			—	—	23.5	0.5	1.5	3	206	26	3.0
17	150	260			—	—	8.3	0.55	1.5	4	254	26	2.4

* This rat had multiple cysticercuses in the liver.

surfaces, their consistency was elastic soft, and their color yellowish red. A tendency to cirrhotic change was never observed either in the external appearance or on the cut surfaces. Histological observations proved complete absence of cirrhosis. The hepatic lobuli remained completely normal, while the interstices were delicate. No cholangiofibrosis, nor any kind of bile duct proliferation was seen. The vasculatures were normal. Neither adenoma nor hepatoma occurred. In one word, the livers remained structurally completely normal (Figg. 10–18).

Group E-F: The findings on the livers of 10 rats in this group varied rather conspicuously. Their weight ranged from 9.7 g to 21.3 g. None appeared normal, and all of them showed at least one adenomatous nodule. Among them only three had nodular lesions, consistent with malignant hepatoma in one and mixed hepatocholangioma in two. Histological study revealed relatively intensive cirrhotic change in all animals associated with cholangiofibrosis and in some, with cholangiocysts, except in three in which the cirrhotic lesions of the liver were

Table 4. Gross findings of the organs (Group E-F—DAB+Hypophesectomy)

Rat No.	Body Weight (g)		Amount of DAB (mg) fed (average)	Liver		Organ Weight						
						Visceral			Internal Secretory			
	Initial	Final		Tumor	Cirrhosis	Liver (g)	Spleen (g)	Kidneys (g)	Hypophysis (mg)	Thymus (mg)	Adrenals (mg)	Testes (g)
1	128	280	549.12	±	+	16.5	0.8	2.6	7	397	37	4.3
2	155	305	527.72	+	+	19.5	1.3	2.2	5	424	32	4.2
3	170	235	533.00	±	++	15.7	1.2	2.0	6	380	38	3.8
4	160	250	563.58	++	++	21.3	1.3	2.0	6	309	41	4.0
5	160	320	570.38	−	+	19.5	1.2	2.2	5	410	28	4.2
6	170	215		−	+	10.0	0.8	1.4	2	252	26	3.0
7	115	165		−	+	9.7	0.8	1.4	2	260	32	3.6
8	135	226	557.76	−	+	10.8	0.8	1.7	3	313	26	3.6
9	120	190		++	++	18.0	2.5	1.5	4	205	32	2.2
10	120	240	557.88	−	+	16.2	1.4	2.0	5	340	45	3.4

Table 5. Histological findings of the livers (Group A—DAB only), Plate XIV

Rat No.	Cholangio-fibrosis	Cirrhosis	Cholangiocyst	Type of Cancer	Adenoma	Parenchymal necrosis	Ceroid pigments	Small round cell group	Hemosiderosis of the Stellate cells
1	++	++	−	Hepatoma	++	−	±	−	±
2	+	++	++	−	+	−	±	−	±
3	++	++	++	−	+	−	±	−	±
4	±	++	−	Hepatoma	++	−	+	±	+
5	±	++	−	Hepatoma	++	−	−	+	+
6	+	++	−	Hepatoma	++	±	−	±	±
7	+	++	+	Hepatoma	++	+	−	+	+
8	+	++	−	Hepatoma	++	−	−	+	±
9	++	++	±	−	++	−	−	+	±
10	++	++	++	−	±	−	−	−	±
11	++	++	+	−	±	−	±	±	+

remarkably slight and only occasional proliferation of the periportal stroma concomitant with mild round cell infiltration was seen. It is of particular interest that the animals, which showed only mild hepatic lesion, had only very little

Table 6. Histological findings of the livers (Group B—DAB+PHP), Plate XV.

Rat No.	Cholangio-fibrosis	Cirrhosis	Cholangiocyst	Type of Cancer	Adenoma	Parenchymal necrosis	Ceroid pigments	Small round cell group	Hemosiderosis of the Stellate cells
1	−	−	−	−	−	−	±	−	−
2	−	−	−	−	−	−	±	±	−
3	−	−	−	−	−	−	±	−	−
4	−	−	−	−	−	−	±	+	±
5	±	−	±	−	−	−	+	−	±
6	−	−	−	−	−	−	±	−	+
7	−	−	−	−	−	−	−	±	+
8	−	−	−	−	−	−	+	−	+
9	−	−	−	−	−	−	±	±	±
10	−	−	−	−	−	−	±	−	−
11	−	−	−	−	−	−	−	±	−
12	−	−	−	−	−	−	−	±	±
13	±	−	−	−	−	−	±	−	±
14	−	−	−	−	−	−	±	±	−
15	−	−	−	−	−	−	−	−	±
16	−	−	−	−	−	−	±	+	+
17	−	−	−	−	−	−	−	−	±

Table 7. Histological findings of the livers (Group E F—DAB+Hypophysectomy) Plate XVI

Rat No.	Cholangio-fibrosis	Cirrhosis	Cholangiocyst	Type of Cancer	Adenoma	Parenchymal necrosis	Ceroid pigments	Small round cell group	Hemosiderosis of the Stellate cells
1	+	++	++	−	+	−	±	−	±
2	++	++	±	Hepatoma	+	−	−	−	±
3	++	++	+	−	+	−	−	±	±
4	++	++	+	Hepatoma Cholangioma	++	−	−	−	±
5	++	++	±	−	+	−	−	−	−
6	+	++	+	−	++	−	−	−	±
7	++	+	+	−	+	−	±	+	±
8	++	+	+	−	++	−	−	−	±
9	++	++	±	Hepatoma Cholangioma	++	±	−	−	±
10	−	++	+	−	+	−	−	−	±

remnants of their anterior pituitary glands after the primary attempt of hypophysectomy. The pituitary weighed 2 mg in Rat Nos. 6 and 7, and 3 mg in No. 8.

Findings on the internal secretory organs:

Pituitary: In Group A, the pituitary ranged between 4 and 9 mg, and averaged 6.54 mg in weight. No gross abnormality was noticed. Small weight of the gland appeared to be associated with small body weight. Histologically, however, neither qualitative nor quantitative difference was observable. The animals of Group B had a lower average weight of the glands of 4.59 mg, ranging from 3 mg to 7 mg. Decrease in the weight of the pituitary glands in Group B appears to be statistically significant. But, whether this is due to PHP dosage in Group B or to the established hepatoma in Group A remains yet to be studied. Grossly and microscopically, the glands appeared not to be different from those of the previous group. In Group E-F, in which complete hypophysectomy was attempted, the majority of the animals survived had the pituitary glands weighing in the range of 4 to 7 mg. They showed no particular sign of previous injury, both grossly and histologically. Three of the animals had pituitaries of 2, 2, and 3 mg, respectively. Although, gross and histological distorsions of their structure were noticed, all three kinds of the anterior gland cells were discernible on microscopic examination. An exact ratio of the cellular populations was not calculated. However, no grave deviation from the normal appeared to be present.

Thymus: In all of the three groups the thymus weighed between 96 and 410 mg. No essential difference in the average weight of this organ appeared to be present among the different groups. Histologically, various grades of fatty metamorphosis were observed in the thymus of every group. No group-associated histologic change has taken place.

Adrenal: Gross appearances of the adrenals were non-remarkable in all the animals of the three groups. Histological changes could not be substantiated. No degeneration occurred in any animal. The weight of the organs per animal averaged 32.8 mg, 26.8 mg and 33.7 mg in the groups A, B and E-F, respectively. Apparently the adrenals were smaller in average in the Group B, in which no hepatic cirrhosis occurred, than in the other groups which showed various grades of cirrhosis. The three rats of the Group E-F, which showed very limited hepatic lesion had similar low average of 28.0 mg.

Testis: The testes appeared grossly normal in all groups. The average weight of the organs in the Groups A, B and E-F were 3.07 g, 2.82 g and 3.63 g respectively. The animals which were partially hypophysectomized had testes averaging 3.40 g in weight. Only slight tendency to reduction of the testicular weight in the Group B was discernible. Histological examination revealed neither suppression nor

activation of spermatogenesis in any animal.

The other organs: The spleen presented no remarkable lesion in all of the three groups, except moderate to marked enlargement in the hepatoma bearing animals. Histologically, the enlargement appeared to be attributable to congestion, deposite of pigment and certain grades of reticulosis. The kidneys were non-remarkable in all groups, in both gross and histologic appearances.

Discussion

The results obtained in the present series, as those of Robertson, Griffin and Richardson's previous experiment, appear to show 100% inhibition in the occurrance of hepatoma in the groups of rats fed simultaneously with DAB and PHP. Histologic specimens revealed, in addition, a complete suppression of even the cirrhotic lesions, which have been believed to be precancerous in the DAB carcinogenesis. The livers were practically normal and not even an adenomatous proliferation occurred.

The mechanism of inhibitory actions of PHP could be analysed along the following lines: either the substance acts through the hormonal system or directly to the liver.

So far as the accumulated clinical knowledge on PHP suggests, the substance may act as a metabolic substitute of an estrogenic hormone. Lacassagne et al., however, found no concrete histocytologic changes in any of the organs concerned in the activity of the sex hormones after an intensive treatment of mice with PHP, although they found diminution in weight of the testicles. As indicated in Table 3, there was a slight decrease in the weight of the pituitary, adrenal, and testicle in Group B in which the rats were fed chronically with DAB and PHP, as compared with in Group A. However, a thorough histologic examination revealed no conclusive difference in those organs of the rats of the two groups. In view of the profound damages to the liver in Group A, a purely hormonal explanation in this concern seems to be unjustified.

Griffin et al. hypophysectomized 26 rats and fed them with 3'-Me-DAB in synthetic diet. Of the 24 rats surviving 14–19 weeks, 19 were found to be completely hypophysectomized. Only one of them showed mild cirrhosis of the liver but no tumor. Richardson et al. further commented that incomplete hypophysectomy in three rats was followed by development of cirrhosis, and that the inhibitory action of hypophysectomy could markedly be diminished by the administration of ACTH and STH. Pituitary gonadotropin and thyrotropin had less effect, while cortisone, testosterone and DOCA were completely ineffective. An assumption was made that changes in lipid distribution in the adrenal cortices might have been related to the inhibitory hormonal mechanism.

In spite of rather extensive histologic studies of the hormonal organs, there has been no statement in their reports concerning the amount of the carcinogen consumed. As to the incorporation of the carcinogen in the hepatic cells, the subject appears not to have been studied. One case among their 19 rats developed cirrhosis despite complete hypophysectomy. The other livers were stated as not significantly changed. There may still be a possibility that low intake of the carcinogen in the surgically treated animals and some changes in the rate of incorporating the dye into the hepatic cells could result in low incidence and retardated appearance of cancer in the liver.

Our Group E-F cannot be regarded as a hypophysectomized series comparable with these of the previous authors. But, marked suppression of cirrhosis, as well as of hepatoma, in three rats in the series, in which only 1/3 or 1/2 of the pituitaries remained, suggests a definite influence of injury to the gland, inasmuch as the carcinogen taken was at high level.

The present result of PHP showing histo-cytologically normal pituitary anterior lobe on one hand, and absolute inhibition of hepatoma and cirrhosis on the other hand, appears to be of interest. If the assumption is correct that no grave functional change can take place without notable structural variation of the hormonal gland, the whole story may be explained more rationally with the working hypothesis that PHP may act not via pituitary, but more directly.

Estrogenic hormones have been known rather as promoting factors in carcinogenesis than as inhibitors. Earlier experience suggested lower incidence of hepatoma in female rats as compared with male. Amano et al. believed an estrogen acted as a weak inhibitor while an androgen as a promotor in DAB carcinogenesis in rats. But, Tsunoda showed reverse results in a small group of animals after a shorter duration of experiments. There are further indications that estrogens can be a weak promotor in experimental carcinogenesis in general.

As PHP is an exact half of stilbesterol in its chemical structure, some assume similar activity of PHP as a metabolic substitute of the estrogen. But, the results obtained in this series are entirely different. As has been shown above in the experimental data, the average amounts of the basic diet consumed were found no lower in Group B and Group E-F than in Group A. These same data also reveal that DAB was no less taken-in in Group B. Consequently, a competition in the hepatic cells between DAB and PHP to form protein bound substances appears to be of significance, if such could actually take place in the rat livers after feeding the combined diet.

A preliminary *in vitro* test was first performed to exclude the possibility of *in vitro* deterioration of DAB in the presence of PHP in sufficient quantity. After DAB, PHP and DAB plus PHP were dissolved in a small quantity of ether-

alcohol a. a. separately, 7 N HCl was added to make a final solution of 1/25000 N of each. Absorption curves of the three kinds of solution were plotted using a Beckman spectrophotometer.

As is shown in Chart 1, the absorption curve of the solution of DAB plus PHP revealed only additive effect of DAB and PHP assessed separately. It seems reasonable to assume that no specific chemical reaction between DAB and PHP occurs *in vitro*.

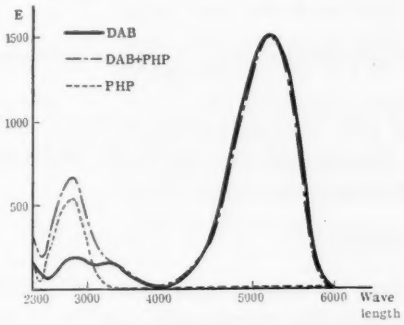

Chart 1. Absorption curves of other-alcohol solutions of DAB, DAB+PHP, and PHP.

Table 8. Protein bound DAB in DAB and DAB+PHP Groups.

Extinctions of the azo dyes extracted from both groups of 100 mg liver powders hydrolysed with 7 N HCl.

	DAB Group	PAB+PHP Group
520 mμ	0.185	0.121
280 mμ	1.330	2.100

As azo dye usually disappears completely from the rat livers after cessation of feeding, a small model experiment was carried out to determine the bound azo dye contained in the hepatic parenchyma after feeding.

Two groups, consisting of 4 rats each, were fed 16 days with the basic diet alone and the basic diet plus PHP respectively, as in the case of Groups A and B in the main experiments. The animals were sacrificed immediately after 16 days, and protein bound azo dyes in both groups of livers were extracted and determined after the technique of Miller and Miller. Dry powders were prepared from the livers of 4 rats in each group, and the quantity of protein bound azo dye in 100 mg of the powders was estimated. As it is demonstrated in Table 8, the livers of the animals fed with DAB plus PHP contained protein bound DAB at the level of 2/3 of the livers from the rats fed DAB only, as determined by extinction at 520 mμ. This diminution in the bound azo-dye could be attributable to the presence of protein conjugated PHP entering into the hepatic cells in competition with DAB. It is not yet certain that all the inhibitory action of PHP on the hepatom induction is entirely due to this competitive ability of the chemical.

Summary and Conclusions

1. p-Hydroxypropiophenone administered orally to a group of seventeen rats fed with 0.06% DAB diet for 160 days exhibited a 100% inhibition of incidence of hepatoma and of cirrhosis.

2. Imperfect hypophysectomy in a group of ten rats appeared to have some inhibiting action in a similar series.

3. The above findings were compared with those in the control group administered with DAB alone.

4. PHP appeared not definitely acting via hormonal system, in view of histological findings of the organs of the animals.

5. Some data are presented to suggest the possibility of a direct action of PHP to the hepatic cells in inhibiting the liver damage and carcinogenesis, although the mechanism involved leaves much to be investigated in the future.

Acknowledgments

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Explanation of Figures

Plate XIV. Group A—DAB only (Cf Table 5)

Fig. 1. Gross appearance of the liver. (Rat 1) (Hepatoma with extensive cirrhosis)
Fig. 2. Gross appearance of the liver. (Rat 6) (Hepatoma with extensive cirrhosis)
Fig. 3. Gross appearance of the liver. (Rat 5) (Hepatoma with extensive cirrhosis)
Fig. 4. Gross appearance of the liver. (Rat 7) (Hepatoma with extensive cirrhosis)
Fig. 5. Gross appearance of the liver. (Rat 11) (Severe cirrhosis)
Fig. 6. Cholangiocysts. (Rat 2) X 50
Fig. 7. Severe cholangiofibrosis. (Rat 3), X 50
Fig. 8. Severe liver cirrhosis. (Rat 7), X 50
Fig. 9. Typical trabecullar hepatoma. (Rat 7), X 100

Plate XV. Group B—DAB+PHP (Cf Table 6)

Fig. 10. Gross appearance of the liver. (Rat 1) (Almost normal, one cysticercus cyst at the right. edge)
Fig. 11. Gross appearance of the liver. (Rat 5) (Almost normal)
Fig. 12. Gross appearance of the liver. (Rat 6) (Almost normal)
Fig. 13. Gross appearance of the liver. (Rat 12) (Almost normal)
Fig. 14. Gross appearance of the liver. (Rat 17) (Almost normal)
Fig. 15. Normal appearance of the liver structure. (Rat 15). X 15
Fig. 16. Very slight cholangiofibrosis which occurred in a few cases. (Rat 13), X 100
Fig. 17. So-called round cell group which occurred in a few cases in Group B. (Rat 12), X 100
Fig. 18. Most intensive fibrosis in the livers of Group B. (Rat 12), X 100

Plate XVI. Group E-F—DAB+Hypophysectomy (Cf. Table 7)

Fig. 19. Gross appearance of the liver in a partially hypophysectomized rat. (Rat 5) (Almost normal but slightly cirrhotic)
Fig. 20. Gross appearance of the liver in a partially hypophysectomized rat. (Rat 8) (Slightly cirrhotic without tumor)
Fig. 21. Gross appearance of the liver. (Rat 8) (Severe cirrhosis with granular surface)
Fig. 22. Gross appearance of the liver. (Rat 4) (Hepatoma nodule to the left)
Fig. 23. Gross appearance of the liver. (Rat 9) (Two hepatoma nodules and severe cirrhosis)
Fig. 24. Relatively normal liver structure in a partially hypophyscectomized rat. (Rat 7), X 50
Fig. 25. Relatively normal appearance of the liver in a partially hypophysectomized rat. (Rat 5), X 50
Fig. 26. Adenomatous hyperplasia with cholangiofibrosis. (Rat 3), X 100
Fig. 27. Typical trabecular hepatoma. (Rat 4), X 100

要　旨

Butter Yellow によるラット肝癌発生に及ぼす p-Hydroxypropiophenone (PHP) の抑制的効果

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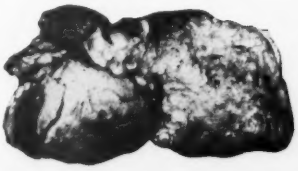
(癌研究所)

DAB 投与によるラット肝癌発生の実験的抑制の研究は，中原等の牛肝粉末以来，多くの業績が見られるが，未だ，特定の機構は確認されていない。一方，Griffin 等は，実験的腫瘍発生における下垂体前葉ホルモンの重要性を強調している。p-Hydroxypropiophenone (PHP) は，Dodd の発見になるが，Lacassagne 等は下垂体の TSH, Gonadotropin の分泌を抑制すると説いている。著者は下垂体関係をめぐる実験的発癌の研究で，DAB によるラット肝癌発生に及ぼす PHP の効果と，hypophysectomy の影響について実験した。PHP は Robertson 等が少数の動物で試みて報告している如く，非常に毒性の低い物質であり，しかも butter-yellow 発癌に対して，100%抑制作用を示めした。このことについて肉眼所見と組織所見を詳述した。この PHP の抑制の機転については，下垂体関係のホルモンの影響を介てしというよりは，むしろ，肝蔵蛋白に対する DAB と PHP の競合にある可能性が強く，これに関する data も示した。

Hypophysectomy は，不完全であったが，部分的に剔出されているものは，その程度に応じて発癌は抑制されていた。



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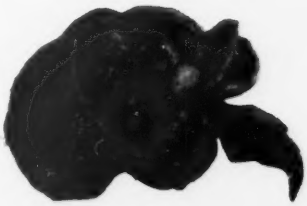

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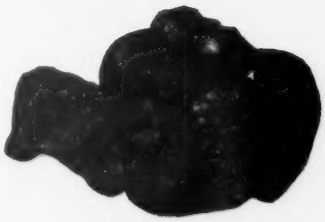

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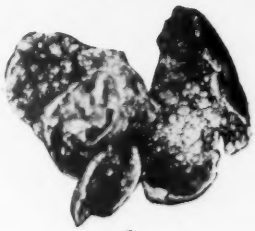

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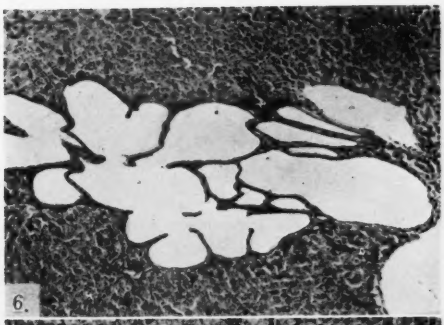

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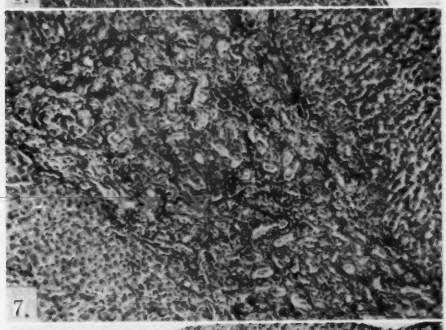

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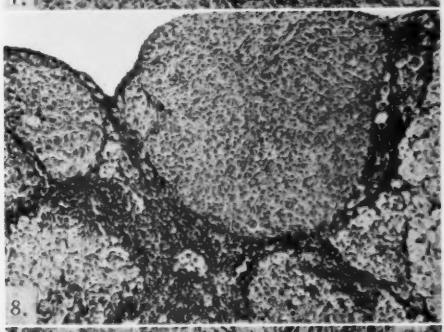

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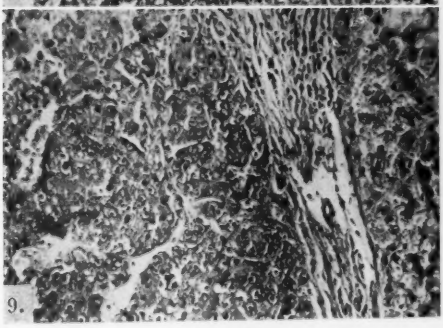

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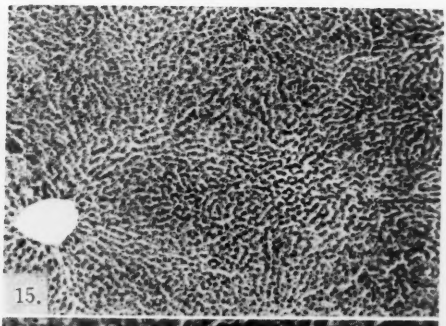

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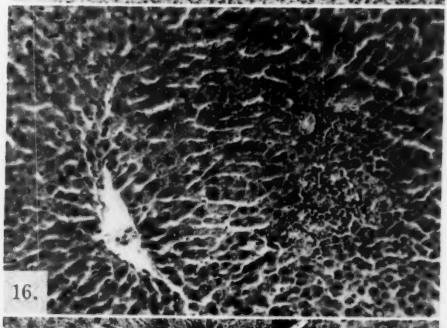

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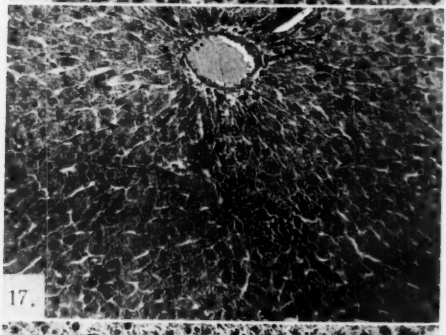

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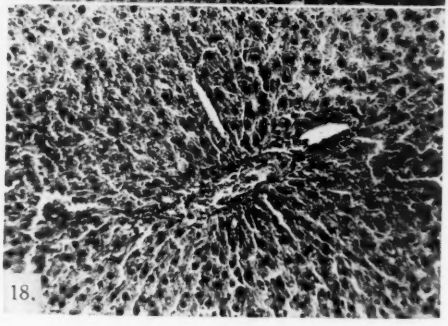

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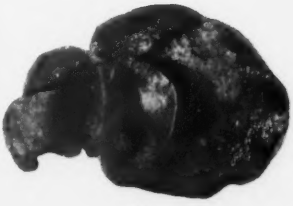

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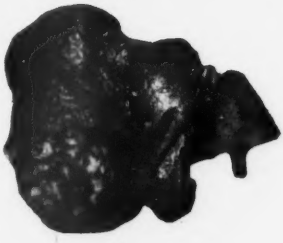

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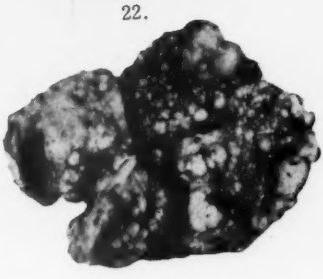

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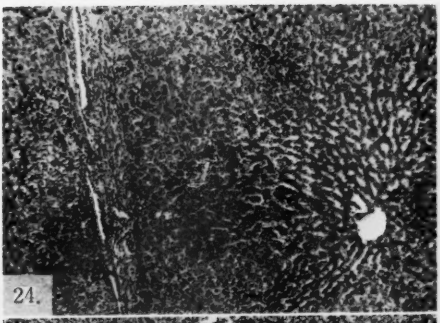

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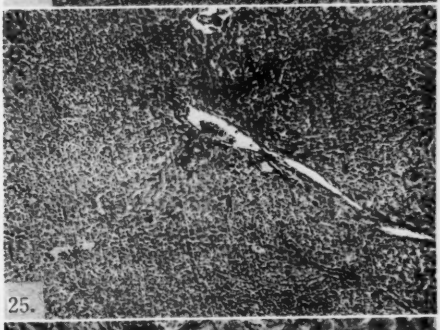

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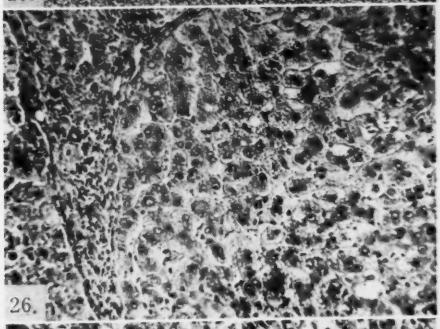

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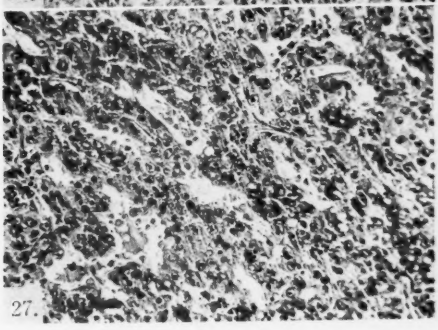

27.

[GANN, 48, 159~168; June, 1957]

DISTRIBUTION OF RADIOACTIVE IRON INJECTED INTO TUMOR-BEARING ANIMALS

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In view of the marked liver catalase depression and anemia that are found in tumor-bearing animals and cancer patients, considerable attention has long been focussed on the disturbance of iron metabolism in them. Many reports have been published regarding iron metabolism in tumor-bearing animals and cancer patients. It was noted that the serum iron level in cancer patients was considerably decreased (1, 2, 3, 4). Intravenously injected Fe^{59} disappeared from plasma more rapidly in cancer patients than in normal human beings (2). It was also reported that liver ferritin content was low in gastric cancer patients (5, 6, 7).

In a previous paper (8), we described that mice bearing NF-carcinoma (mammary carcinoma) or NF-sarcoma (fibrosarcoma), showed remarkable catalase depression but their liver ferritin content was at about the same level as that of normal control mice. In these cases, NF-carcinoma and NF-sarcoma were completely free from necrosis and hemorrhage.

In the present paper, we give data on the distribution of radioactive iron injected into rats bearing Rhodamine fibrosarcoma. The tumor in this case was relatively free from necrosis and hemorrhage. In these tumor-bearing animals, there were liver catalase depression, anemia with reticulocytosis, lowered iron storage in liver, accelerated uptake of radioactive iron into erythrocytes hemoglobin and decline in incorporation of radioactive iron into liver iron compounds. These results completely agree with those of Greenfield and Price in the experiments using rats bearing lymphosarcoma (9, 10). At the time of this writing, the details of their experimental results have not been available to us, however.

EXPERIMENTAL

Male albino rats (Saitama Strain) were used throughout these experiments. They were kept in brass wire cages and were maintained on the usual laboratory diet of wheat containing 3 percent dried fish powder. Fresh vagetables were given once a week. Additional iron was not loaded on them throughout these experiments. Transplantable Rhodamine sarcoma (11) was used as tumor, in which hemorrhagic and necrotic portion was relatively small, even when the tumor was

of considerable size. When the tumor reached a certain size, the radioactive iron was intraperitoneally injected. Twenty-four hours after the injection, animals were anaesthetized with injection of sodium pentobarbiturate (100 mg per kg of body weight), and blood was withdrawn from the abdominal aorta using the syringe containing heparin. Plasma was separated by contrifugation. Red cells were washed twice with the same volume of physiological saline. Crystalline hemin was prepared from these washed red cells by the method of Chu and Chu (12). Plasma iron was determined by the method of Noda and Higuchi using nitroso-R with a slight modification (13). Liver was removed and non-hemin, and ferritin were fractionated by the method of Yoneyama and Konno (14). In some cases, kidney, spleen and bone marrow of both femurs were removed and the total iron contents were assayed. Moreover, the radioactivities excreted for the twenty-four hours in urine and feces were also determined in some cases. Iron in tissues was determined by o-phenanthroline method. For the determination of radioactivity, the iron was precipitated with the solution of ammonium-polysulfide after adding 200 μg of iron as carrier. Iron sulfide was filtered on filter paper. The hemoglobin content was assayed colorimetrically after mixing blood with 0.1 percent sodium carbonate (15). Reticulocytes in peripheral blood were counted according to the method of Kämmere (16). Catalase activity in liver was determined by the method of Euler and Josephson (17).

Radioactive iron (Fe^{59}) was purchased from the Atomic Energy Commission in the United States. The specific activity of this sample was 1906 mc/g. Solution containing radioactive iron was diluted to a suitable concentration with 1/10 M acetate buffer (pH 5.0) and 5-10 μc was intraperitoneally injected into rats. As the specific activity of iron was very high, the actually injected dose of iron was smaller than 5 μg. It seemed that with this low level of iron, iron metabolism will not be affected in these animals. In order to define the rate of uptake of radioactive iron into various iron compounds, the relative specific activity was calculated through dividing the specific activity by the body weight and by total injected rodioactivity.

At all stages of these experiments, the analysis of tumor-bearing animals and of control animals were done in parallel.

Results

In Table 1, the various data of experimental animals are presented. The average liver catalase activity in tumor bearing animals was 60.5% of that in control animals. The average hemoglobin contents in blood were 12.2 and 7.5 g per 100 ml in control and tumor-bearing animals, respectively. Remarkable reticulocytosis in tumor-bearing animals is notable, although with considerable

Table 1. Various data on the experimental animals

	No.	Sex	Body Weight (g)	Liver Weight (g)	Spleen Weight (g)	Kidney Weight (g)	Hemo-globin Content (g/dl)	Liver Catalase Activity (k/10mg/min)	Reticulo-cytes (‰)	Tumor Weight (g)	Days after Trans-plantation
Control Animals	1	male	230	4.38	—	—	—	—	—	—	—
	2	male	245	4.75	—	—	—	—	—	—	—
	3	male	207	4.54	—	—	13.6	—	—	—	—
	4	male	270	5.65	—	—	12.8	—	—	—	—
	5	male	168	6.00	0.98	1.28	11.9	0.241	86	—	—
	6	male	163	4.95	1.06	1.16	11.3	0.263	34	—	—
	7	male	150	4.70	0.45	1.00	12.6	0.245	18	—	—
	8	male	145	4.50	0.40	1.05	11.2	0.192	26	—	—
	Average		198	5.06	0.96	1.12	12.2	0.235	41	—	—
Tumor-bearing Animals	1	male	390 (305)	0.25	—	—	7.4	—	—	85	31
	2	male	254 (162)	8.15	—	—	8.6	—	—	92	31
	3	male	170 (120)	5.85	0.81	1.20	8.1	0.236	158	50	21
	4	male	168 (128)	5.65	0.59	1.16	10.0	0.168	97	40	21
	5	male	220 (168)	8.20	2.15	1.50	6.9	0.080	238	52	19
	6	male	188 (135)	5.50	2.65	1.20	4.5	0.049	525	53	19
	7	male	225 (176)	9.30	1.80	1.45	6.7	0.179	162	49	19
	Average		231 (171)	6.23	1.60	1.30	7.5	0.142	295	60	

individual variation.

The full data concerning the iron concentration in the various tissues and radioactivity incorporated into them are given in Tables 2 and 3. Specific activity was calculated. In Charts 1 and 2, the iron concentration and relative specific

Chart 1. Iron Concentration of Various Tissues in Normal and Tumor-Bearing Animals

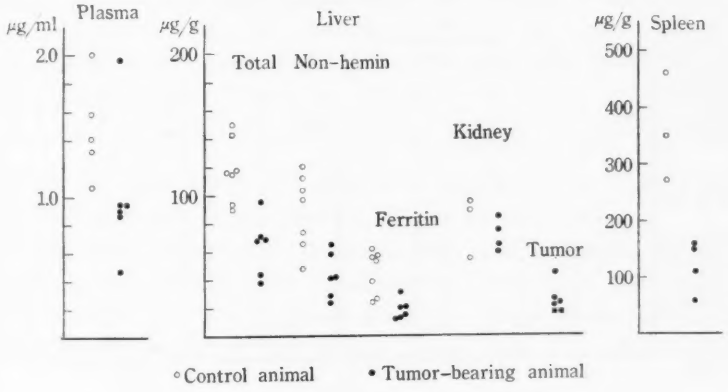


Table 2. Iron concentration and radioactivity of various tissues in control animals.

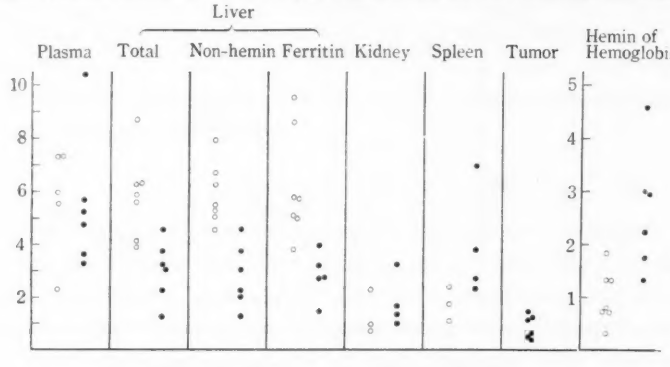
Animal No. Body Weight (g)	C1 230				C2 245				C3 207				C4 270			
Injected Total Act. (cpm)	4.04×10^6				4.04×10^6				1.50×10^6				1.50×10^6			
	Feμg/g	cpm/g	S	R	Feμg/g	cpm/g	S	R	Feμg/g	cpm/g	S	R	Feμg/g	cpm/g	S	R
Plasma	—	112	—	—	—	142	—	—	2.0	33	16.5	2.27	1.41	58	41.2	7.29
Liver Total	115	11900	104	5.95	116	11890	103	6.25	143	4010	28.1	3.88	118	3840	32.5	5.84
Liver Nonhemin	112	10250	92	5.25	97	8730	90	5.45	104	3790	36.5	5.03	74	3260	44.0	7.90
Liver Ferritin	56	5660	101	5.76	53	4420	84	5.09	62	1720	27.8	3.83	39	1866	47.8	8.60
Kindney	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Spleen	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Hemin of Blood (cpm per 200 μg of hemin Fe)	—	6030	32	1.84	—	2640	13.2	0.80	—	489	2.44	0.33	—	814	4.07	0.73

Animal No. Body Weight (g)	C5 168				C6 163				C7 150			
Injected Total Act. (cpm)	1.36×10^6				1.36×10^6				1.80×10^6			
	Feμg/g	cpm/g	S	R	Feμg/g	cpm/g	S	R	Feμg/g	cpm/g	S	R
Plasma	1.07	64	39.8	7.29	1.58	73	46.3	5.55	1.32	94	71.2	5.94
Liver Total	90	3010	33.4	4.12	94	4940	52.7	6.31	150	15720	104.8	8.70
Liver Nonhemin	48	1775	37.0	4.56	66	3440	52.1	6.25	120	1150	80.6	6.71
Liver Ferritin	24	970	40.4	4.98	26	2072	79.6	9.53	57	3900	68.4	5.70
Kindney	94.5	528	5.6	0.69	88.5	694	7.84	0.94	55	1480	26.9	2.24
Spleen	272	3840	14.1	1.74	461	240	9.2	1.10	352	9950	28.3	2.36
Hemin of Blood (cpm per 200 μg of hemin Fe)	—	1806	9.03	1.13	—	1806	9.03	1.13	—	1209	6.04	0.72

S: specific activity

R: relative specific activity $= \dfrac{\text{Specific activity (cpm/g)}\times\text{Body weight (g)}}{\text{Total injected dose (cpm)}}\times10^3$

Chart 2. Relative Specific Activity of Ironin Various Tissues of Normal and Tumor-Bearing Animals



◦Control animal •Tumor-bearing animal

Table 3. Iron concentration and radioactivity of various tissues in tumor-bearing animals

Animal No.		T-1				T-2				T-3			
Body Weight (g)		390				254				170			
Injected Total Act. (cpm)		1.50×10^6				1.50×10^6				1.36×10^6			
		Feμg/g	cpm/g	S	R	Feμg/g	cpm/g	S	R	Feμg/g	cpm/g	S	R
Plasma		0.90	18	20.1	5.20	0.94	20	21.3	3.60	0.87	33	38.0	4.75
Liver	Total	71	905	12.8	3.32	96	696	7.3	1.24	44	845	19.3	2.41
	Non-hemin	65	505	7.7	2.00	59	432	7.3	1.24	29	523	18.0	2.25
	Ferritin	31	322	10.4	2.70	20	170	8.5	1.44	13	334	25.7	3.21
Kidney		—	—	—	—	—	—	—	—	85	696	8.2	1.02
Spleen		—	—	—	—	—	—	—	—	150	3270	21.8	2.72
Blood (cmp per 200μg of Hemin Fe)		—	2259	11.30	2.94	—	2625	13.13	2.23	—	2747	13.74	1.72
Tumor	hemorrhagic	22	45	2.0	0.52	23	45	2.0	0.39	17	155	9.1	1.14
	Hemorrhagic												

Animal No.		T-4				T-6				T-7			
Body Weight (g)		168				188				225			
Injected Total Act. (cpm)		1.36×10^6				1.80×10^6				1.80×10^6			
		Feμg/g	cpm/g	S	R	Feμg/g	cpm/g	S	R	Feμg/g	cpm/g	S	R
Plasma		0.94	43	45.8	5.65	0.47	74	157	16.4	1.96	50	26.3	3.29
Liver	Total	69	1349	19.5	2.41	38	1399	36.8	3.85	68	2205	32.4	4.05
	Non-hemin	41	1005	24.5	3.03	24	1040	43.5	4.55	42	1255	29.8	3.73
	Ferritin	15	478	31.9	3.94	12	314	26.2	2.74	21	—	—	—
Kidney		65	864	13.2	1.64	59	1840	30.9	3.23	74	805	10.8	1.35
Spleen		158	2961	18.8	2.32	58	3870	66.7	6.97	110	3350	30.4	3.80
Blood (cpm per 200μg of Hemin Fe)		—	2130	10.7	1.32	—	8750	43.8	4.58	—	4800	24.0	3.00
Tumor	hemorrhagic	45	250	5.4	0.66	13.6	190	14.0	1.47	26.3	265	10.0	1.25
	Hemorrhagic					303	8135	26.8	2.84	57.9	160	2.8	0.35

S: specific activity

R: relative specific activity $= \dfrac{\text{Specific activity (cpm/g)} \times \text{Body weight (g)}}{\text{Total injected dose (cpm)}} \times 10^3$

activity as the measures of iron uptake are illustreated. In Table 4 the average iron concentration and relative specific activity are summarized.

The decrease in concentration of plasma iron, total non-hemin and ferritin iron in liver, and total iron in spleen were observed. In kidney, the concentration of iron did not change. In tumor tissue the concentration of iron was relatively low, but the level of iron concentration in hemorrhagic part of tumor was considerably high.

Table 4. The average values of iron concentration and relative specific activity in various tissues

	Control animals		Tumor-bearing animals	
	iron content (μg/g)	Relative specific activity	iron content (μg/g)	Relative specific activity
Plasma	1.48	5.69	1.01	6.48
Liver				
total	118	5.68	64.3	2.85
non-hemin	89	5.88	43.0	2.73
ferritin	45.2	6.41	18.8	2.80
Kidney	79.5	1.32	71.1	1.81
Spleen	362	1.76	119	3.96
Hemin of blood	—	0.91	—	2.63
Tumor	—	—	24.5	0.91

The relative specific activity of iron in the various tissues decreased in the order of plasma, liver, spleen, kidney, hemin of blood, and tumor. It must be pointed out that the relative specific activity of tumor iron was considerably low. The relative specific activity of plasma iron was almost the same in both groups. Concerning the liver iron compounds, the relative specific activity in tumor-bearing animals was about one-half of that in control animals.

The relative specific activity of total, non-hemin and ferritin iron almost did not differ in each group. Contrary to the case of liver, the relative specific activity of crystalline hemin prepared from red cells in tumor-bearing animals was three times higher than that in control animals. The relative specific activity of spleen also increased in tumor-bearing animals.

Table 5. Data on Bone Marrow

	C-7	T-6	T-7
Total iron in bone marrow of both femur (μg)	3.9	1.4	2.3
Total radioactivity in them (cpm)	392	114	136
Specific activity	100	81	59
Relative specific activity	8.32	8.50	7.40

In Table 5 the data concerning the bone marrow are presented. Although the number of experiments are few, it seems certain that the relative specific activity of bone marrow does not greatly differ between tumor-bearing and control animals. C-5 and C-6 in control animals and T-3 and T-4 in tumor-bearing animals excreted in urine and feces respectively 400 cpm and 646 cpm of injected radioactive iron in 24 hours. This means that there is no great difference between tumor-bearing and control animals regarding the excretion of injected iron.

Discussion

From the above described experimental results, it is evident that the uptake of radioactive iron in erythrocyte hemoglobin increased but the uptake of that in the liver iron compounds, on the contrary, decreased when radioactive iron was injected into tumor-bearing animals, in which liver catalase depression and anemia with reticulocytosis were established.

The iron concentration of tumor tissues is relatively lower than that of kidney, liver and spleen, but the total amount of iron in tumor tissue was larger than that in liver. This conclusion was drawn from the following calculations. Average weight of tumor tissues, liver of control animals and liver of tumor-bearing animals were respectively 60 g, 5.08 g and 6.23 g. And since the average iron concentration in these three tissues were respectively 24.5 μg, 118 μg and 64.3 μg, the total iron amont contained in these three tissues were estimated to be about 1.5 mg, 0.6 mg, and 0.4 mg respectively. It is certain that the amount of deposited iron in tumor tissue was larger than the amount of storaged iron in liver. This facts is in agreement with the results reported by Greenfield and Price (9, 10). If we consider that the iron content in hemorrhagic part of tumor is higher, the total iron in tumor tissue must be more than 1.5 mg. We found that a large part of iron in tumor was non-hemin iron (29).

It was reported that the volume of blood in the normal rat was 5.3 ml per 100 g body weight (18). So in our experiments, average blood volumes in normal and tumor-bearing animals were estimated to be about 10.5 ml and 9.0 ml. So the total hemoglobin was about 1.3 g and 0.7 g in normal and tumor-bearing animals. Since the iron content in hemoglobin was 0.34 percent, and the most of iron in erythrocytes comes from hemoglobin, the total iron contained in blood was calculated to be 4.4 mg and 2.4 mg in normal and tumor-bearing animals, respectively.

It must be pointed out that, in the tumor-bearing animals, the iron of hemoglobin in blood and liver was transfered into tumor tissue. Tumor-bearing animals are in the state of iron deficiency. Greenfield and Price (9, 10) described that when erythrocytes labeled with Fe^{59} were injected into animals, a large portion of the Fe^{59} lost from the blood stream was recovered in the tumor. When erythrocytes labeled with Cr^{51} were injected into animals, the most of the activity lost from the blood were found in tumor, but when lysed erythrocytes labeled with Cr^{51} were injected, the most of them were found in liver and spleen. Anemia appeared as the results of the transfering of iron into tumor tissues. Shortening of the life span of erythrocytes was reported in cancer patients (19-22) by many workers but not in tumor-bearing animals (23). Increased uptake of

iron shows the increased compensatory hemoglobin synthesis. This agrees with the reticulocytosis. As the results of increased uptake of iron into erythrocytes, the uptake of radioactive iron into liver fraction was depressed.

Catalase activity depression was partly explanined by this iron deficiency in liver, since Gubler *et al.* (24) recently reported that an iron deficient diet caused liver catalase depression without effect on cytochrome c oxidase in swine. But in their case, the iron content in liver of iron deficient animals reached to one-nineteenth of control, but even in such severe iron-deficient liver, its catalase activity amounted to 62 percent of control. As we reported in our previous paper (8) that liver catalase decreased in the case with no changes in the liver iron storage, catalase depression was regarded as being caused by another mechanism, namely, the inhibition of iron insertion into porphyrin by toxohormone action.

The findings in the present experiments agree with the fact that free porphyrin accumulated in liver in tumor bearing animals (25) and also with Fukuoka and Nakahara's report on the counteraction of iron against toxohormone (26). Free porphyrin increase in erythrocytes (27) is explained partly through the inhibited insertion mechanism of iron into porphyrin and by the decrease of available iron concentration in erythrocytes. The extensive experiments of Schwartz *et al.* revealed that as the rate of erythropoiesis and hemoglobin synthesis increase, there is the proportional increase in the amount of erythrocyte coproporphyrin, which is roughly in proportion to the reticulocyte percentage (28). In the blood of tumor-bearing animals, as presented in our previous reports in this journal (25), the type of erythrocyte porphyrin which is increased is almost entirely composeed of protoporphyrin. It is known that as erythropoiesis is impaired by the presence of iron deficiency and lead poisoning, erythrocyte protoporphyrin increases. It is expected that iron administration to tumor-bearing animals might affect the increased level of erythrocyte protoporphyrin. The experiments along this line is now being considered in this laboratory.

Summary

Radioactive iron was injected into control and tumor-bearing rats. The tumor used was Rhodamine sarcoma, which is relatively free from necrosis and hemorrrhage. The iron amount and radioactivity were determined in tumor, plasma, hemoglobin, liver, kidney and spleen 24 hours after the injection.

The concentration of iron in liver and plasma decreased in tumor-bearing animals. The incorporation of radioactive iron into hemoglobin increased and the incorporation of radioactive iron into liver decreased in tumor-bearing animals. It was estimated that the total iron contained in tumor-tissue was

larger than the total iron amount in liver.

Distribution of iron in tumor-bearing animals was discussed in relation to anemia, liver catalase depression and porphyrin acummulation in liver and red cells in the case of tumor-bearing animals.

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要　旨

担癌動物に注射した放射性鉄の分布について

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(癌研究所)

放射性鉄をローダミン肉腫を移植したラットに腹腔内注射して，24 時間後に殺し，腫瘍，肝，血液その他の鉄量を測定し，また放射能を測定した。

担癌動物は貧血，網状赤血球増加，肝カタラーゼ活性低下があり，肝鉄量，血漿鉄の減少が著明である。腫瘍組織の鉄濃度は低いが，腫瘍塊中の総鉄量は肝より多い。担癌動物の赤血球よりとった結晶ヘミンの鉄の相対比放射能は正常動物の約 3 倍高く，肝の総鉄，ノン・ヘミン鉄，フエリチン鉄の相対比放射能は正常動物の約 1/2 である。

これらの事実から，担癌動物における鉄代謝の様相を理解し，あわせて肝カタラーゼ活性低下，貧血，ポルフィリン蓄積等に関連して考按した。　(文部省科学研究費による)

[GANN, 48, 169~180 ; June, 1957]

FURTHER STUDIES ON THE REDUCTION OF TRIPHENYL-TETRAZOLIUM CHLORIDE BY THE SUCCINIC DEHYDROGENASE COMPLEX

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Many workers (1, 2, 3) used TTC (triphenyltetrazolium chloride) or its derivatives for the determination of succinic dehydrogenase of various tissues since Kun and Abood (4) first applied it for the determination of succinic dehydrogenase in the liver and kidney homogenates of rats. In a previous paper, we (5) described that the pigeon breast muscle succinoxidase prepared by the method of Keilin and Hartree (6) did not reduce TTC in the presence of succinate as substrate while the same preparation showed a high succinoxidase activity with the manometric method of Schneider and Potter (7). In that paper (5) we reported the fact that TTC reduction was restored intensively in the above described system by the addition of crude coenzyme A preparation. We concluded that a certain cofactor was required for TTC reduction by succinic dehydrogenase complex. This conclusion was in agreement with the reports of Martin *et al.*, who used tuberculous guinea pig kidney (8, 9, 10). We fractionated crude coenzyme A preparation by means of Dowex-1 resin and obtained one peak which was active for the restoration of TTC reduction.

In the course of further purification and isolation of this cofactor, we found that Keilin and Hartree's succinoxidase purified by repeated isoelectric precipitation lost the ability to reduce TTC in the presence of succinate as substrate even with addition of crude coenzyme A as cofactor. This seemed to us very strange at first, but in later experiments we could demonstrate that protein component(s) contained in rat liver could restore TTC-reducion activity in the system composed of purified pigeon breast muscle succinoxidase, crude coenzyme A, succinate and TTC. In this paper we describe the experimental results regarding this phenomenon.

METHODS

Kun's original method was used for TTC reduction. The triphenyl formazan, which was formed in the aerobic reaction for a certain time at 37°C without shaking, was extracted with acetone or with ethyl acetate in the case of addition of methylene blue. Formazan was determined by Beckman spectrophotometer

and data were expressed as extinctions at 480mμ.

Keilin and Hartree's method was carried out to prepare the succinoxidase from pigeon breast muscle. In some cases, the isoelectric precipitation, as described in their paper, was repeated two or three times. These preparations were diluted to the suitable concentration with 1/100 M phosphate buffer, pH 7.0, before using.

Rat liver was homogenized with 10 volumes of 1/100M phosphate buffer, pH 7.0, and nuclei were removed by the centrifugation at 3000 rpm for 3 minutes. In this paper, the term "homogenate" means this homogenate free from nuclei. And the particles were centrifuged down at 12,000 g for 20 minutes. Supernatant was separated and packed particles were resuspended in the original volume of phosphate buffer. This method was based on Bril's paper (11). Dialysis of supernatant was carried out against 1/100M phosphate buffer, pH 7.0, at 4°C for 24 hours.

In parts of these experiments, the manometric method of succinoxidase of Schneider and Potter (7) and the manometric assay of succinic dehydrogenase using methylene blue and cyanide (12) were applied.

DPN of 90 percent purity was purchased from the Nutritional Biochemical Corporation, U. S. A. Cytochrome c was prepared by the method of Keilin and Hartree (13) from the heart muscle of horse. Crude coenzyme A was prepared from hog liver as described in the previous paper (5).

Results

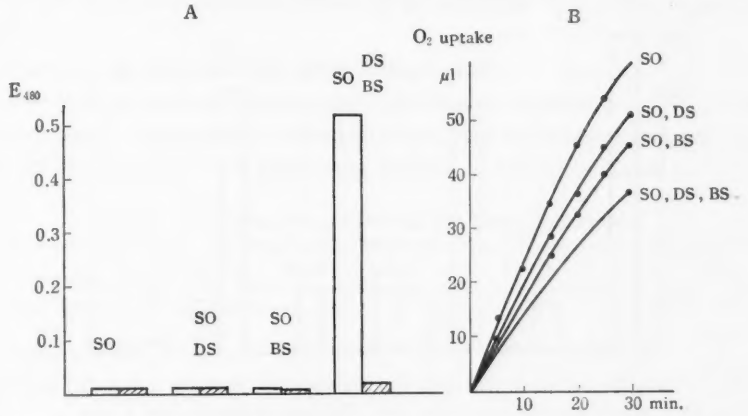
TTC Reduction by Purified Pigeon Breast Muscle Succinoxidase with Succinate.

The purified pigeon breast muscle succinoxidase failed to reduce TTC in the presence of succinate as substrate, but the TTC reducing activity was restored by adding the combination of boiled rat liver supernatant and dialyzed rat liver supernatant. The boiled or dialyzed supernatant alone had no effect on the restoration. This means that cofactor required for TTC reduction was present in boiled supernatant and the some protein factor required for TTC reduction was present in the dialyzed supernatan. This purified preparation of pigeon breast muscle succinoxidase showed a strong succinic dehydrogenase activity with the method of Slater. The addition of boiled supernatant and/or dialyzed supernatant did not enhance the oxygen uptake but inhibited it slightly. This mechanism of inhibition was not explained. These results are shown in Chart 1.

TTC Reduction by Cellular Particles of Rat Liver with Succinate.

The results of the experiments using the particles of rat liver as succinoxidase are shown in Chart 2. Homogenate free from nuclei could reduce TTC in the presence of succinate. But particles resuspended in the same concentration scarcely reduced TTC under the same condition. When the supernatant was added, particles regained the capacity to reduce TTC. As in the case of pigeon breast muscle

Chart 1. TTC Reduction by Purified Pigeon Breast Muscle Succinoxidase



A. SO : 0.5ml of pigeon breast muscle succinoxidase suspension, activity of which roughly corresponds to that of 10% rat liver homogenate by Slater's method.
DS : 0.5ml. dialyzed supernatant from 10% rat liver homogenate.
BS : 0.5ml, boiled supernatant from 10% rat liver homogenate.
1/5M Na succinate 0.5ml, 0.2% TTC 0.5ml, 1/10M phosphate buffer, pH 7.0, 1.0ml.
Each tube contains final volume 3,5ml, 30 minutes at 37°C, aerobic, extracted with 6.5ml of acetone.

B. SO : 0.5ml of pigeon breast muscle succinoxidase suspension in Exp. A.
DO : 0.5ml, same with Exp. A.
BS : 0.5ml, same with Exp. A.
0.01M methylene blue 0.3ml, 0.1M KCN 0.3ml, 0.4M Na succinate 0.2ml, 0.6M phosphate buffer, pH 7.2,0.5ml. Each vessel contains final volume 3.0ml.

succinoxidase the addition of the boiled supernatant or dialyzed supernatant alone had no effect. The combination of boiled supernatant and dialyzed supernatant was as active as the original supernatant. In all the charts, the shaded bars represent the experiments without addition of succinate. In this experimental condition the homogenate could not reduce TTC at all in the presence of fumarate or malate at the same concentration level with succinate. This is represented in Table 1.

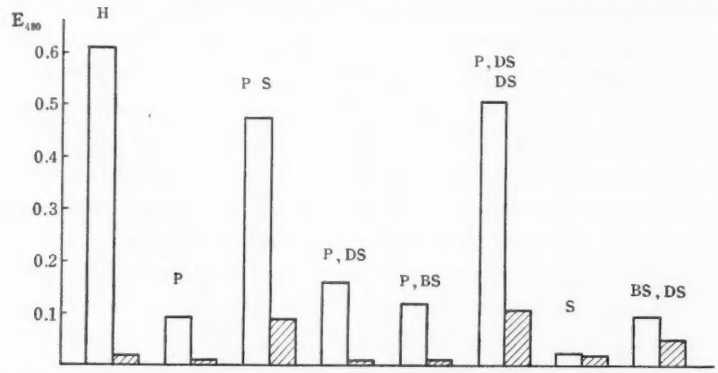
Table 1. TTC reduction by Homogenate of Rat Liver with Fumarate and Malate.

	E_{480}
Succinate	925
Fumarate	92
Malate	112
None	19

Homogenate (10%) 1.0 ml, 1/5M substrates 0.5 ml, 0.2% TTC 0.5ml, 1/10M phosphate buffer pH 7.0 1.0ml.

Homogenate and resuspended particles showed the same activity with the

Chart 2. TTC Reduction by Cellular Fractions of Rat Liver in the Presence of Succinate.



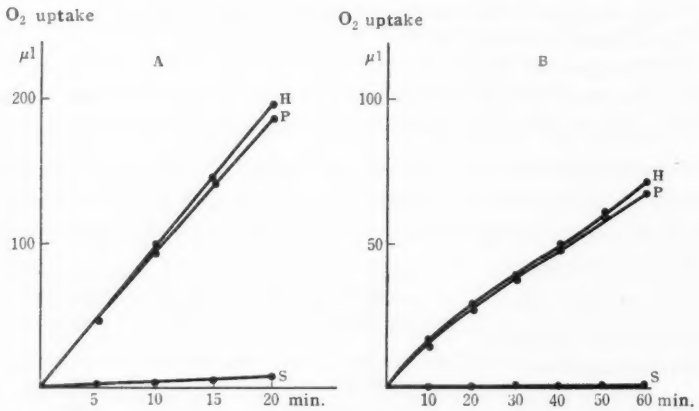
H : homogenate(10%) of rat liver, 1.0ml, P : resuspended particles 1.0ml. S : supernatnat 1.0ml, BS : boiled supernatant 1.0ml. DS : dialyzed supernatnant, 1.0ml.

1/5M Na succinate in 1/10M phosphate buffer, pH 7.0 0.5ml, 0.2% TTC in 1/10M phosphate buffer, pH 7.0, 0.5ml

Final volume 4.6ml, For 30 min. at 37°C, extracted with 6.0ml of acetone.

Each bar represents the average value of five individual rat livers.

Chart 3. Succinic Dehydrogenase Activity in Celluar Fractions determined with Manometric Methods.



A. Schneider and Potter's method

10% rat liver homogenate 0.5ml ; 4×10^{-3}M $AlCl_3$ 0.3ml ; 4×10^{-3}M $CaCl_2$ 0.3ml ; 0.5M Na succinate, 0.3ml ; 1/10M phosphate buffer, pH 7.4, 1.0ml ; 1×10^{-4} M cytochrome c, 0.4ml. Final volume 3.0ml.

B. Slater's method

10% homogenate. 0.5ml ; 0.01M KCN, 0.3ml; 0.4M Na succinate 0.2ml ; 0.6M phosphate buffer, pH 7.2, 0.5ml. Final volume 3.0ml.

Schneider and Potter's method as with Slater's method. It was clear that almost all the activity in homogenate was recovered in particles. These results are given in Chart 3.

Fractionation of Dialyzed Rat Liver Supernatant.

It is clear that the cofactor and a certain protein are required for TTC reduction by succinoxidase. This protein contained in rat liver supernatant was precipitated between 33-50% saturation of ammonium sulfate, as shown in Table 2.

Table 2. Fractionation of Dialyzed Rat Liver Supernatant.

Addition		E_{480}
Dialyzed Rat Liver Supernatant	0.0 ml	147
	0.1	240
	0.3	352
	0.5	519
	1.0	789
Fraction Precipitated between 0-33% Saturation of $(NH_4)_2SO_4$	1.0*	120
Fraction Precipitated between 33-50%	1.0*	545
Fraction Precipitated between 50-60%	1.0*	187
Fraction Precipitated between 60-45%	1.0*	197

*Precipitate was dissolved in 1/100M phosphate buffer, dialyzed, and was adjusted to original volume.

All tubes contain: Purified pigeon breast muscle succinoxidase suspension, 1.0 ml; Crude caenzyme A solution 0.5 ml; 0.2% TTC 0.5 ml; 1/5 M Na succinate 0.5 ml; TTC and Na succinate was dissolved in 1/10M phosphate buffer pH 7.2; 30 min. at 37°C, extracted with 6.5 ml cold acetone.

Boiled Extract, Crude Coenzyme A and Dialyzed Supernatant of Rat Liver as a Rate Limiting Factor in TTC Reduction Reaction.

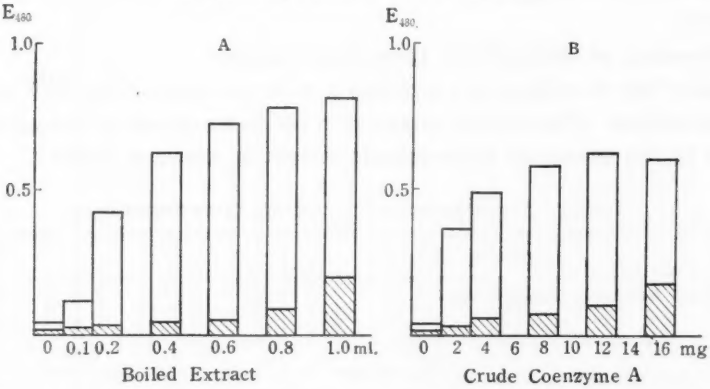
In Chart 4 is shown the experiment in which the excess amount of dialyzed supernatant and various amounts of boiled extract or crude coenzyme A preparation were added. In this case, the amount of boiled extract may be rate limiting factor within a certain range for TTC reduction. The assay of amount or activity of cofactor with this system is quite within the bounds of possibility.

In Chart 5 is illustrated the experiments in which the excess amount of boiled extract and the various amounts of dialyzed supernatant were added. In this system, the dialyzed supernatant became a rate limiting factor.

DPN and Other Preparations as Cofactor.

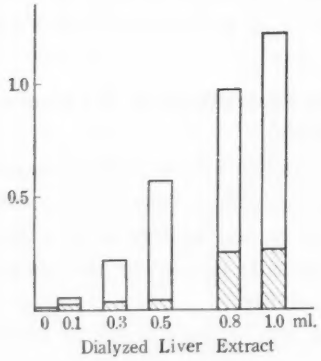
DPN could replace the boiled extract of liver or crude coenzyme A when the rat liver particles were used as succinoxidase. On the other hand DPN could hardly replace them when the purified pigeon breast muscle succinoxidase was used as succinoxidase. In the latter case, crude coenzyme A preparation, crude

Chart 4. Boiled Extract of Rat Liver and Crude Coenzyme A Preparation as a Rate Limiting Factor for TTC Reduction.



Purified pigeon breast muscle succinoxidase suspension 0.5 ml; 0.2% TTC in 1/10M phosphate buffer, pH 7.2, 0.5ml; 1/5M Na succinate in 1/10M phsophate buffer, pH 7.2, 0.5ml; dialyzed rat liver supernatant 0.8ml; Boiled extract was prepared as follows: same volume of water was added to tissue and homogenized and boiled for 10min. and supernatant was obtained after centrifugation for 20 min. at 37°C. Total volume 3.5ml, extracted with 6.5ml of acetone.

Chart 5. Dialyzed Rat Liver Supernatant as a Rate Limiting Factor



Purified pigeon breast muscle succinoxidase suspension, 0.5ml; 0.2% TTC in 1/10M phsophate buffer, pH 7.2, 0.5ml; 1/5M sodium succinate in 1/10M phosphate buffer, pH 7.4, 0.5ml; Boiled extract (prepared as described in foot note for Chart 4.) 1.0ml, Various amounts of dialyzed supernatant fractions from 10% rat liver homogenate were added. For 20 min, at 37°C. Totol volume 3.5ml.

TPN fraction prepared from pig liver, and barium soluble, ethanol insoluble fraction of acid soluble fraction of beef liver could replace the boiled extract but not barium insoluble fraction of acid soluble fraction of beef liver. The above described findings support that the active principle is correlated with the dinucleotide fractions. The response of DPN in the rat liver particles experiment agreed with the reports of Bril (11) and Waldschmidt-Leitz (18). These results are presented in

Table 3.

Table 3. DPN and Other Preparations as Cofactor.

	Addition as cofactor	with succinate	without succinate
Pigeon Breast Muscle Succinoxidase	Crude Coenzyme A	0.345	0.131
	Acid soluble fraction		
	Barium insoluble	0.015	0.003
	Barium soluble, ethanol insoluble	0.224	0.048
	TPN fraction	0.576	——
	DPN (NBI)	0.098	0.023
	None	0.028	0.005
Rat Liver Particles	DPN	0.561	0.093
	None	0.101	0.020

Each tube contains: 0.5ml of pigeon breast muscle succinoxidase or rat liver particles equivalent to 10% homogenate; 0.2% TTC 0.5ml; 1/5M Na succinate 0.5ml; dialyzed rat liver supernatant, 0.5ml; TTC and Na succinate was dissolved in 1/10M phosphate buffer, pH 7.2.

Addition was 5 mg in each tube.

30min. at 37°C, extracted with 6.5ml of acetone.

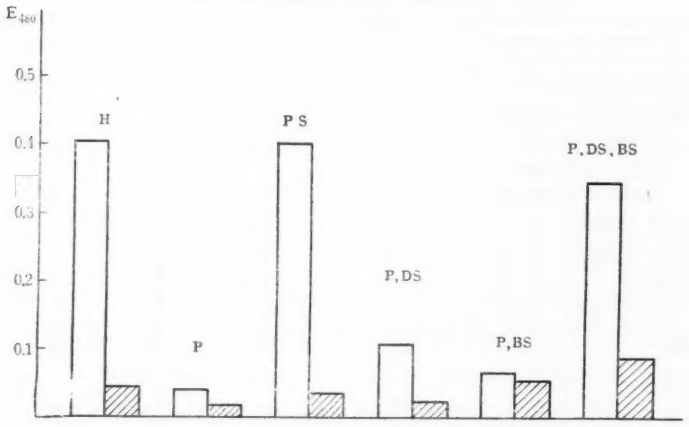
TTC Redution by Cellular Particles of Rat Liver with Reduced Cytochrome C.

When we used the reduced cytochrome c instead of succinate as hydrogen donor, rat liver particles could reduce TTC. In the case of the reducing of cytochrome c, ascorbate was used. Hydroquinone also gave similar results. As in the case of using succinate, resuspended particle failed to reduce TTC in the presence of reduced cytochrome c, while the homogenate reduced TTC in the same condition. Addition of dialyzed supernatant and boiled supernatant of rat liver to particles could restore the TTC reducing activity of particles. These results are given in Chart 6. At this condition, ascorbate itself did not reduce TTC.

The Effect of Methylene Blue and Cyanide Inhibition.

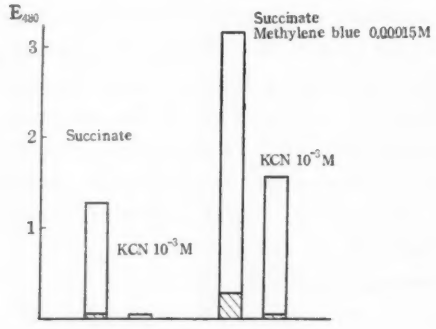
TTC reduction by rat liver homogenate was enhanced by the addition of methylene blue of 0.001M final concentration, as already reported by Zöllner *et al.* (14). Cyanide at the concentration of 10^{-3}M inhibited the TTC reduction by succinate but the inhibition was lesser with the addition of methylene blue. This was shown in Chart 7. Moreover it was confirmed that when particle was used as succinic dehydrogenase, methylene blue activation did not require the protein component.

Chart 6. TTC Reduction by Cellular Fraction of Rat Liver in the Presence of Reduced Cytochrome c.



H : homogenate (10%) of rat liver 1.0ml., P : resupended particles 1.0ml., S : supernatant 1.0ml., BS : boiled supernatant 1.0ml., DS : dialyzed supernatant 1.0ml. 0.2% TTC in 1/10M phsophate buffer pH 7.0 1.0ml ; 1.5×10^{-3}M cytochrome c 0.1ml ; 0.114M Na ascorbate 0.4ml.; Total volume 4.0ml. for 20 min. at 37°C. This chart represents the average value of four experiments.

Chart 7. The Effect of Methylene Blue and Cyanide



10% rat liver homogenate 1.0ml ; 1/5M Na succinate in 1/10M phosphate buffer, pH 7.0, 0.5ml ; 0.2% TTC in 1/10M phosphate buffer, pH 7.0, 0.5ml. Final concentration of methylene blue 0.00015M. Final concentration of KCN 0.001M.

Total volume 3.0ml, 30min. at 37°C, extracted with 7.0ml of ethyl acetate.

Discussion

It was suspected that flavoprotein may be the immediate electron donor for TTC reduction from the experiment on *E. coli* and yeast by Brodie and Gots (15, 16). Kun also supported this view from his experiment using amino acid oxidase and the combination of glycolytic enzyme system with rat liver mitochondria or isobutanol extract of mitochondria. But, for the TTC reduction by succinic de-

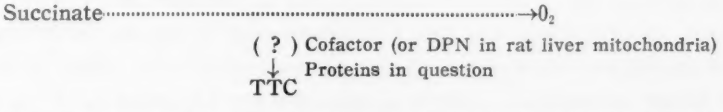
hydrogenase complex, the level, at which TTC was reduced, was not yet determined.

In our previous paper (5) and in the papers of Martin *et al.* (8, 9, 10), the requirement of cofactor for TTC reduction by succinic dehydrogenase complex was confirmed. The nature of that cofactor was not yet finally determined.

As described in this paper, the purified pigeon breast muscle succinoxidase or rat liver mitochondria could not efficiently reduce TTC by succinate even with the addition of boiled supernatant as cofactor. When native protein in rat liver supernatant was added, TTC reduction was restored. Whether this protein factor is a specific one for electron transport or not remains as a problem to be solved. This active protein was contained also in kidney, spleen and heart. The nature and the quantitative distribution in various organs of this active protein are being studied in our laboratory.

When the rat liver particles ware used, DPN could replace cofactor. This agrees with the report of Bril (11). Waldschmidt-Leitz (18) also reported that diluted mouse liver homogenate could not reduce TTC by succinate but it reduces TTC by succinate with the boiled extract of liver or DPN. He suggested the dissociation of enzyme-coenzyme system. However, the mechanism of TTC reduction stimulated by DPN was not clarified. But we cannot completely neglect the possibility that TTC reduction stimulated by DPN was caused by the DPNH produced with malic dehydrogenase *via* citric acid cycle, although the TTC reduction in rat liver homogenate was very weak with the addition of fumarate or malate at the same concentration as the succinate without DPN. The restoring activity contained in the boiled extract of rat liver cannot be explained by the DPN, since DPN concentration in it was very low. It was therefore suspected that the active principle beside DPN is present. The possibility must be considered that impurity of nucleotide contained in DPN preparation may be essentially active. Hoch *at el.* (19) reported that TTC reduction of rat liver mitochondria with succinate was enhanced by the addition of ATP. In the case of pigeon breast muscle in our experiment ATP was not at all effective and DPN showed only a slight effect.

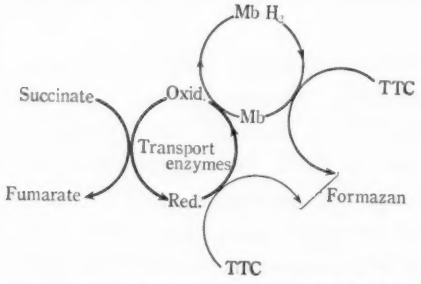
The electron transport to TTC *via* the above mentioned cofactor and proteins from a certain level in the succinoxidase system may be illustrated in the following scheme:



On the other hand, reduced cytochrome c instead of succinate added to rat liver homogenate could reduce TTC. This finding suggests the possibility that TTC is coupled at the level of cytochrome c oxidase. Experiment using cytochrome c

oxidase fragmented from mitochondria will settle this question. And in this case, too, the mitochondria alone could not efficiently reduce TTC, although most of cytochrome c oxidase was known to be recovered in particles (20) and our own experiment showed that 80% of homogenate cytochrome c oxidase was recovered in paticles by Schneider and Potter's method. The combination of dialyzed and boiled supernatant was effective in restoring TTC reduction. Each one, per se, was not effective.

Zöllner (14) reported that methylene blue greatly enhances the TTC reduction by mouse liver homogenate in the presence of succinate, and presented the following scheme:



Besides, it is known that methylene blue enhances TTC reduction by fatty acid oxidizing enzymes (12). In our experiments using rat liver homogenate, TTC reduction by succinate in aerobic condition was strongly inhibited by cyanide while the TTC reduction enhanced by the addition of methylene blue in the presence of succinate was less inhibited. Moreover, the enhancement by methylene blue was of not dependent on the presence of the above mentioned protein components.

From these findings, we present the following scheme ragarding TTC reduction by succinate:

Succinate·····Cytochrome b·····→Cytochrome c oxidase·····→0_2

↓ Mb　　(?)　Cofactor

TTC　　↓　Protein in question

TTC

Recently Kamin *et al.* (22) reported that the heat labile factor ("KS"), which was contained in guinea pig liver and beef kidney water homogenate, was sedimentable between 4000–12000 g and was unable by itself to reduce NT, could stimulate NT reduction by succinate in the dialyzed homogenate of guinea pig kidney. This heat labile factor ("KS") may correspond to the above mentioned protein in our experiments. Kamin stated also that this protein could be replaced by cytochrome c and that cyanide and antimycin A abolish neotetrazolium (NT) reduction by succinate in kidney homogenate. They suggested that the TTC reduction by succinate took place at the level of cytochrome c oxidase.

Apart from the interest in the mechanism of TTC reduction, it is important to consider that TTC reduction was dependent on many factors besides the succinic

dehydrogenase when succinic dehydrogenase activities were compared among the various different kinds of tissues and cellular fractions.

Summary

Pigeon breast muscle succinoxidase purified by repeated isoelectric precipitation, could not reduce TTC in the presence of succinate even though the excess amount of boiled extract of liver or crude coenzyme A as cofactor was added. And TTC reducing activity was restored by the addition of dialyzed supernatant of rat liver homogenate.

It was concluded that a certain protein component, which is contained in rat liver supernatant and has no succinic dehydrogenase activity by itself, was required for TTC reduction by succinoxidase with cofactor.

Rat liver particles could not efficiently reduce TTC in the presence of succinate, but the addition of boiled supernatant and dialyzed supernatant could restore the TTC reducing activity. The addition of each one, per se, could not restore the activity.

The system for the determination of cofactor activity was suggested because the amount of boiled extract or crude coenzyme A became a rate limiting factor under the addition of the excess amount of dialyzed rat liver supernatant.

Rat liver homogenate could reduce TTC in the presence of reduced cytochrome c. In this case, also, the cofactor and protein component were required for efficient TTC reduction by rat liver particles.

Methylene blue could enhance the TTC reduction in rat liver homogenate by succinate. Cyanide strongly inhibited TTC reduction in rat liver homogenate by succinate, but inhibited it less in the addition of methylene blue.

From these results, the mechanism of TTC reduction by succinoxidase were discussed.

Our hearty thanks are due to Dr. Waro Nakahara for his kind interest and encouragement.

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要　旨

コハク酸還元酵素によるTTC還元機構に関する研究

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先にわれわれは，鳩胸筋より調整したコハク酸酸化酵素は十分に検圧法で活性があるにもかかわらずTTCを還元することができない。そして，それに粗コエンチームA標品を加えるとTTC還元がおこることをみとめ，それをイオン交換樹脂でわけた。

その後その物質を追求する途次に，また別の新事実を見出した。

等電沈澱をくり返した鳩胸筋コハク酸酸化酵素はコエンチームA標品を十分加えてもTTC還元がおこらなくなるがこれに，ラット肝の上清分劃中の蛋白を加えると十分なTTC還元が起るようになる。すなわち，コハク酸を基質とした，コハク酸酸化酵素によるTTCの還元には未知の助酵素のほかに，肝上清にふくまれる蛋白成分が必要なのである。

同様のことがラット肝のミトコンドリアをコハク酸酸化酵素として用いた場合にもみとめられた。鳩胸筋のコハク酸酸化酵素を用いると，この未知助酵素または，蛋白成分を Rate limiting になるような system を作ることができる。

また還元したチトクロームcを加えるとラットモジエネートでもTTC還元がおこること，この場合もミトコンドリアでは十分TTC還元がおこらないことをみとめた。メチレンブルーを加えるとコハク酸を基質としたTTC還元は促進されるがこれはチアン阻害を受け難い。メチレンブルーを加えない時にはチアン阻害は著しい。

以上からコハク酸酸化酵素によるTTC還元には助酵素のほかに肝上清中の蛋白の必要なことおよび，TTCがチトクローム酸化酵素のレベルで共軛する可能性を考察した。

（文部省科学研究費による）

[GANN, Vol. 48, 181~188, June, 1957]

STUDY ON THE NATURE OF BINDING OF DIMETHYLAMINOAZOBENZENE WITH LIVER PROTEINS IN RATS FED THE CARCINOGEN
I. ISOLATION AND PURIFICATION OF THE AMINO ACID BOUND AMINOAZO DYE

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INTRODUCTION

It has been discovered by Kinosita (1) that the long period of administration per os of dimethylaminoazobenzene (DAB) causes hepatoma in rats. In advance of the outcome of the hepatoma which usually occurs after 5 to 6 months of feeding, the binding of a metabolic intermediate of DAB with liver proteins was observed by Millers et al. (2, 3, 4), who then proposed the so-called "protein deletion" theory (5) as the etiology of certain cancers. Evidences of such a binding of carcinogens with proteins *in vivo* are also obtained with polycyclic aromatic hydrocarbons (5, 6, 7) and aminofluorene derivatives (8, 9). The chemical nature of the binding with the protein is not elucidated yet except with a few carcinogenic hydrocarbons (10, 11, 12).

Following the pioneering works mostly done by Millers and their co-workers, the authors started to investigate the chemical and biochemical nature of DAB binding with rat liver protein. The livers of albino rats fed DAB for about one month were digested with a very active extracelluar protease* prepared from the *Streptomyces* culture (13). Out of the hydrolysate, peptides containing aminoazo dye were extracted and purified.

EXPERIMENTAL

Material: Male albino rats (body weight about 100 g.) were used. The diet of carrot and rice which is coated with DAB dissolved in olive oil (0.06 per cent DAB in rice) was fed ad libitum**. After a month the animals were sacrificed. The livers were removed immediately and homogenized with Waring blender. Appropriate amount of trichloroacetic acid was added under mechanical stirring, then warmed up in a boiling water bath for 5 minutes. The slightly reddish coagulate

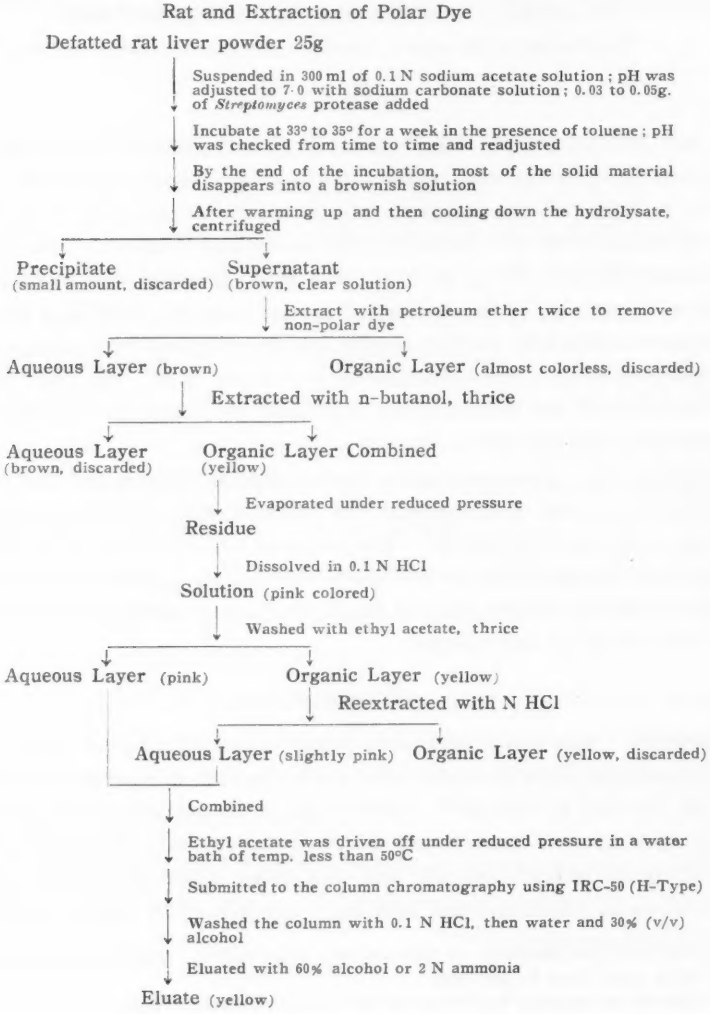
* The substrate specificity of this protease is not limited. The degree of hydrolysis usually goes up to more than 90 per cent.

** Care of the animals was taken at the Cancer Institute, Tokyo.

was filtered and washed with acetone. The residue was further extracted with Soxhlet apparatus using a mixture of methanol and chloroform (1 : 1) for 10 hours. After the extract became colorless, the residue was taken out and filtered, and washed with ether. The material was then dried in a vacuum desiccater and pulverized. This can be submitted to the proteolytic hydrolysis and the extraction of the dye containing peptide thereafter.

The Enzymatic Hydrolysis of Rat Liver Powder Fed DAB and the Extraction

Table 1. An Example of Enzymatic Hydrolysis of Liver of DAB Fed Rat and Extraction of Polar Dye


Defatted rat liver powder 25g
Suspended in 300 ml of 0.1 N sodium acetate solution ; pH was adjusted to 7·0 with sodium carbonate solution ; 0.03 to 0.05g. of Streptomyces protease added
Incubate at 33° to 35° for a week in the presence of toluene ; pH was checked from time to time and readjusted
By the end of the incubation, most of the solid material disappears into a brownish solution
After warming up and then cooling down the hydrolysate, centrifuged
Precipitate (small amount, discarded)
Supernatant (brown, clear solution)
Extract with petroleum ether twice to remove non-polar dye
Aqueous Layer (brown)
Organic Layer (almost colorless, discarded)
Extracted with n-butanol, thrice
Aqueous Layer (brown, discarded)
Organic Layer Combined (yellow)
Evaporated under reduced pressure
Residue
Dissolved in 0.1 N HCl
Solution (pink colored)
Washed with ethyl acetate, thrice
Aqueous Layer (pink)
Organic Layer (yellow)
Reextracted with N HCl
Aqueous Layer (slightly pink)
Organic Layer (yellow, discarded)
Combined
Ethyl acetate was driven off under reduced pressure in a water bath of temp. less than 50°C
Submitted to the column chromatography using IRC-50 (H-Type)
Washed the column with 0.1 N HCl, then water and 30% (v/v) alcohol
Eluated with 60% alcohol or 2 N ammonia
Eluate (yellow)

of "Polar Dye": An example of the general procedure is illustrated in Table 1. Yields of the peptide containing aminoazo dye vary remarkably depending on the groups of the animals, but usually about 8 γ of polar dye (expressed as DAB) per rat was obtained under our experimental conditions. This yield was higher than that by the Millers' alcoholic alkali hydrolysis (2), mostly due to its mild experimental conditions. The spectrum of the polar dye thus prepared is shown in Fig. 1.

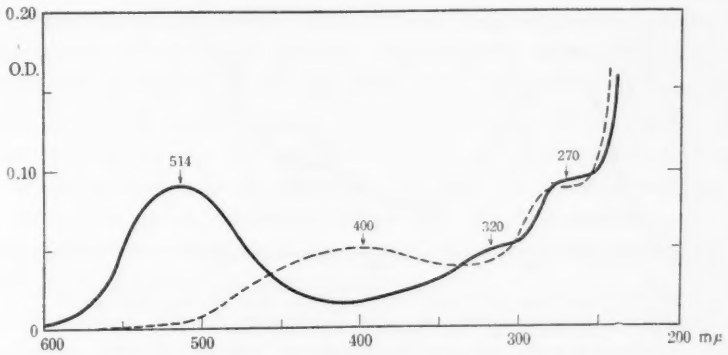


Fig. 1. Spectra of Enzymatically Prepared Polar Dye
Solid curve: in ca. 0.5 N Hydrochloric Acid
Dotted curve: in ca. 0.1 N Ammonia

Alkali Hydrolysis of Enzymatically Prepared Polar Dye: The polar dye prepared enzymatically as described in Table 1 appears to contain peptides consisting of several amino acids. In order to break off the peptide bonds without decomposing aminoazo dye moiety, the enzymatically prepared polar dye was submitted to the alkali hydrolysis. But the result showed unfortunately the partial destruction of the dye moiety as described later. Twice as much volume of 4.5 N sodium hydroxide solution was added to an aqueous solution of the enzymatically prepared polar dye. The mixture was refluxed on a water bath for 6 to 10 hours. After the hydrolysis, it was extracted with n-butanol. The butanol extract was washed successively with water, 0.1 N hydrochloric acid, water, 0.1 N ammonia and finally water. After the final washing, the butanol layer was separated. The butanol was evaporated under reduced pressure. The residue was dissolved in 0.1 N hydrochloric acid and the pink solution thus obtained was submitted to the column chromatography as described in Table 1. The spectrum is shown in Fig. 2.

Amino Acids in Enzymatically Prepared Polar Dye: The polar dye was hydrolyzed in the presence of 6 N hydrochloric acid for 12 hours. The amino acids thus liberated were assayed according to the dinitrophenylation technique (14). The amino acids identified were as follows in the order of the amounts detected:

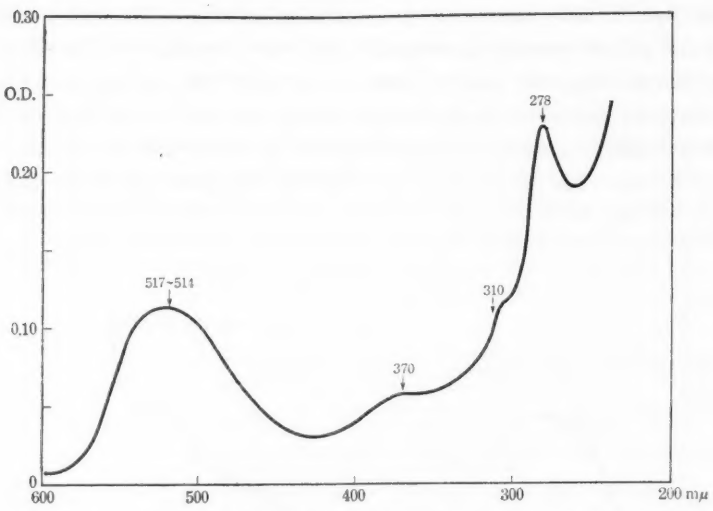


Fig. 2. Spectrum of Alkali Treated Polar Dye in Alcoholic 0.66 N Hydrochloric Acid (Alcohol : 40 %, v/v)

Major components ; Pro, Gly, Phe, Lys*, Leu, Val.

Minor components ; Asp, Ser, Ala, Glu.

The treatments of the polar dye with the crystalline carboxypeptidase or crystalline pepsin revealed the liberation of leucine and phenylalanine. If the extinction coefficient of the polar dye is assumed to be identical with that of DAB, though it may not necessarily be so, the average number of the amino acids attached to one mole of aminoazo dye is about 10. The repetition of the enzymatic hydrolysis of the once enzymatically prepared polar dye using the same *Streptomyces* protease revealed the further liberation of considerable amounts of amino acids out of the polar dye, thus shortening the peptides chain of the polar dye.

Spectrophotometrical Assay of Polar Dye : The highly purified polar dye solution thus prepared was assayed spectrophotometrically using Beckman Spectrophotometer Model DU. The spectra of the enzymatically prepared polar dye and further alkali treated polar dye are shown in Figs. 1 and 2. The structually uncorrelated increase in absorption at 370 mμ of the alkali treated polar dye may be due to the partially destroyed aminoazo dye moiety of the polar dye, since DAB similary treated with alkali showed a new absoptions at this wave length. The absorption at 514 mμ to 517 mμ is completely due to the aminoazo dye moiety of the polar dye because there is negligible amount of absorption at 514 mμ in the

* Lysine was found only in the polar dye prepared from the particulate fraction of the rat liver, while not detected from the supernatant fraction.

alkaline or neutral media. (See Fig. 1) No need of basal diet correction (3) is obvious with our samples of the polar dye.

The Yields and Properties of Alkali Treated Polar Dye: The liver of rats fed DAB was divided into two parts. Each of them was hydrolyzed with alkali and the protease, respectively. Alkali hydrolysis was carried out in the same way as described by Millers et al; 50 ml of 4.5 N potassium hydroxide and 20 ml of ethanol were mixed with 0.6 g of the liver powder and the mixture was refluxed in a boiling water bath for 20 hours. On the other hand, 0.6 g of the same sample was mixed with 0.01 g of the protease. The condition of the hydrolysis and the extraction was the same as described above. The yields of polar dyes were 3.4 γ and 5.6 γ, (expressed as DAB) respectively. So the yield by the alkali hydrolysis is 60 per cent of that by the enzymatic hydrolysis. The difference in the yield seems to be ascribable to the loss due to the partial decomposition of the dye moiety during the alkali hydrolysis. When the polar dye prepared enzymatically was hydrolyzed with alcohol-alkali, the 40 per cent of the dye moiety was proved to be decomposed during the alkali hydrolysis.

The difference in the properties between the enzymatically prepared and the alkali treated polar dye is that the latter did not give any significant amino acid upon hydrolysis with 6 N hydrocholric acid, while the former gave several kinds of amino acids. Other properties, such as solubility, stability and so on were almost similar. It may be assumed that the alkali treated polar dye contains only one amino acid, which can not be separated from the dye moiety without causing serious damage upon the both parts of the molecule.

Dinitrophenylation of Alkali Treated Polar Dye: To examine the presence of amino radical in the alkali treated polare dye, 2,4-dinitrobenzene sulfonic acid (DNBS) (15, 16) was put into reaction with the polar dye. This reagent is known to react only with amino group of amino acids (17). The alkali treated polar dye and about five hundreds times as much of DNBS were mixed in a carbonate buffer (M/10, pH 10), and incubated at 35° for 12 hours. After it was extracted with ether, the aqueous fraction was acidified with 2 N hydrochloric acid. The color of the aqueous layer changed into pink on acidification. This indicated that the aminoazo dye moiety remained unchanged in this fraction. The dinitrophenylated polar dye thus prepared can be extracted with ether from the acidic solution but the original polar dye can not. The presence of amino group in the alkali treated dye may be assumed.

In another experiment, the alkali treated polar dye was put into reaction with 2,4-dinitrofluorobenzene (DNFB) in 66 per cent alcoholic aqueous solution in the presence of excess of sodium bicarbonate. DNFB is known to react with amino group as well as hydroxyl of tyrosine, imidazol of histidine, and sulfhydryl of cysteine

(14). After several hours of incubation, the reaction mixture was diluted with water and extracted with ether. The aqueous layer was separated and acidified. It became colorless upon acidification, not pink as in the case of dinitrophenylation by DNBS. Though the aqueous solution was submitted to the paper chromatography, only a spot of dinitrophenol was identified. Apparently the polar dye derivative was extracted with ether from the alkaline solution. This result may suggest that at least two DNP-radicals were introduced to the polar dye. The speculative compound, however, was not identified yet. In order to check the reactivity of aminoazo dye with the dinitrophenylating reagents, DAB was tested and no reaction was observed.

Discussion

The results of the dinitrophenylation may suggest the presence of one amino group and another reactive radical in the alkali treated polar dye.

The amino acids concerned can be tyrosine, histidine or cysteine. Tyrosine, however, seems to be the most probable amino acid among them at the present time, mainly from the following reasons:

The absorption at 270 mμ to 280 mμ, which seems to be too big to be expected from aminoazo dye moiety, was always observed on the spectra of the polar dyes.

Moreover attempts have been done to synthesize the polar dye-like compounds from aminoazo dyes, amino acids, and formaldehyde which is considered an intermediate of the oxidative metabolism of N-methyl radicals of DAB. Out of various amino acids tested, only tyrosine, histidine and tryptophan gave synthetic compounds similar to the polar dye with aminoazobenzene or monomethylaminoazobenzene, but not with DAB, in the presence of excess formaldehyde. Cysteine and other amino acids failed to produce any appreciable amount of stable polar dye. The absorption spectra of the natural polar dye resembled that of the synthetic polar dye consisting of tyrosine, aminoazobenzene and formaldehyde. The detail of these experiments will be reported in a separated paper.

The polar dye prepared both enzymatically and with alkali are rather stable in aqeous hydrochloric acid solutions. At room temperature the solution can be stored for 1 to 2 months without any significant change in the spectra, while the polar dye which was reported to be formed during the incubation of 3-methyl-4-monomethylaminoazobenzene, reduced glutathione and fortified rat liver homogenates, was shown to be very unstable in acid media (18). The stability of the synthetic histidine polar dye was much less compared to that of the synthetic tyrosine polar dye.

The pH dependence of the polar dye is very different from the original DAB

as to their color change. The color changes from pink to yellow between pH 1.1 and pH 2.9 with the natural polar dye, while the original DAB shows its characteristic color change in the higher pH region (pH 2.9 to pH 4.0). The decreased proton affinity of the azo group of the polar dye is obvious.

The authors are inclined to assume the tyrosine-aminoazobenzene combination as the most probable model for the polar dye from the data accumulated up to the present time.

Recently the combination of an amino acid with DAB at position 2 has been suggested (19). Although the authors feel that they have not enough data either to support or to deny this possibility at the present, the introduction of such a big molecule like amino acid at the position 2 seems to be less plausible, considering the steric complexity of the reaction.

Summary

A new preparation method of the polar dye from defatted liver powder of rats fed DAB for a month has been described. The absorption spectra of the most purified sample of the polar dye up to date have been shown, which have absorption maximum at 514 to 517 mμ, 270 to 280 mμ and sometimes slight shoulder at 310 to 320 mμ in acid media. The absorption maximum was observed at 400 mμ and 280 mμ in alkaline media. The data accumulated so far seem to make aminoazobenzene-tyrosine binding the most probable model of the polar dye.

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要　旨

アミノアゾベンゼンの肝臓蛋白との結合に関する研究
第一報　アミノ酸と結合した色素体の抽出並びに精製について

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ヂメチルアミノアゾベンゼン (DAB) を一カ月投与した白鼠肝の脱脂粉末より，高度に精製されたアミノ酸と結合した色素体の抽出法を研究した。放線菌培養液より作られる強力プロテアーゼで予め肝蛋白を水解し，ブタノール抽出，醋エチ洗滌を行ったのち IRC-50 (H 型) でカラムクロマトを行うことによって高度に精製された含アミノ酸色素体を容易にうることができる。このものをさらにアルカリで処理すると，恐らくアミノ酸一個と結合したと考えられる色素体を得る。このものについて，吸収スペクトル，変色 pH 領域，酸性における安定性，DNP-化に対する挙動等の性質を調べた結果，並びにアミノアゾ色素，アミノ酸及びフォルマリンより合成的に作られる類似化合物の性質を比較した結果，色素と結合したアミノ酸としてチロシンがもっとも可能性が多いことを結論した。
(文部省科学研究費による)

[GANN, 48, 189~197 ; June, 1957]

STUDIES ON THE NUCLEIC ACID METABOLISM OF HUMAN MAMMARY CARCINOMA, WITH SPECIAL REFERENCE TO THE EFFECT OF SEX HORMONE

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INTRODUCTION

During the course of studies on the pathogenesis of human mammary carcinoma, the questions arose as to whether the metabolic pattern of mammary carcinoma is different from that of adenosis or fibroadenoma. In order to find some key-points of this problem, the metabolisms of phospholipids and ribonucleic acid were studied by means of *in vitro* or *in vivo* incorporation of ^{32}P into these fractions.

The authors found that the amount of phospholipids in carcinoma tissue as well as the *in vitro* turnover rate of ^{32}P in the ribonucleic acid of carcinoma were higher than those in adenosis or fibroadenoma, whereas the preliminary administration of male hormone was followed by a decrease of the ^{32}P-incorporation into ribonucleic acid of carcinoma. The *in vitro* addition of 10^{-3} M 8-azaguanine also decreased remarkably the ^{32}P-incorporation into ribonucleic acid of carcinoma tissue.

EXPERIMENTAL

1) Materials.

Experimental materials were supplied from the Department of Surgery, Mitsui Kosei Hospital. In the case of *in vivo* experiments, the patients were injected with 250 μc of ^{32}P, two hours prior to the surgical operation. About one gramm of surgically removed mammary tissue was immediately freezed with solid carbon dioxide and sliced by Stadie-Riggs type slicer.

The hormone therapy was carried out by means of the following four series: (1) Administration of Dihydrotestosterone (Stanaprol "Pfeizer") in a dose of 50 mg for 8 days, (2) Administration of Testostrerone propiorate (Enarmon "Teizo") in a dose of 20 mg for 8 days, (3) Administration of Estradiol benzoate

(Ovahormone "Teizo") 40,000 units for 10 days, and (4) Administration of Para-hydroxypropiophenone (PHP) in a dose of 1 g for 10 days.

2) **Method for in vitro incubation.**

The sliced tissue was incubated at 37°C for two hours in 25 ml of the following incubation media under oxygen. The composition of the incubation medium is given in moles: In 25 ml of Krebs-Ringer phosphate buffer (without Ca^{++}, pH 7.4), sodium pyruvate 0.02, glucose 0.02 and $Na_2\ ^{32}PO_4$ 50 μc. The possibility of contamination of samples by adsorbed ^{32}P was checked by a paralell experiment ("O-time" experiment) in which admixture was made of tissue and radioactive substrate with immediate addition of perchloric acid.

In a series of experiments in which the effect of 8-azaguanine was observed, the drug was added in 10^{-3} M or 2×10^{-3} M concentration.

3) **Extraction of phospholipids and ribonucleic acid.**

In the case *in vivo* experiments, extirpated tissues were homogenized with cold 4% perchloric acid in a Waring-blender. In the case of *in vitro* experiments, equal volume of cold 8% perchloric acid was added to the incubation medium. The both solutions were clarified by centrifugation, and the residues were washed, with centrifugation, successively with cold 4% perchloric acid (three times), cold 4% perchloric acid with 0.004 M Na H_2PO_4 as carrier (twice), and cold 2% perchloric acid (three times). The phospholipid fraction was extracted with cold 80% methanol (twice) then twice at 60°C for 3 minutes with chloroform-methanol (1 : 1); the supernatant solutions were finally combined.

The nucleic acids were extracted twice with hot 10% NaCl for fifteen minutes and then twice for sixty minutes, then combined supernatant solutions with 3 volumes of 95% ethanol, overnignt at 0° to yield a residue containing the sodium nucleate. The sodium nucleate was digested at 37°C for 18 hours with 1 N NaOH. The degrated ribonucleic acid was separated from deoxyribonucleic acid by acidification of the solution with HCl, followed by the addition of 2 volumes of ethanol. The deoxyribonucleic acid was collected by centrifugation. The supernatant liquid containing ribonucleic acid was used for the determination of amount and radioactivity.

4) **Quantitative determination of the total and radioactive phosphorus in phospholipid and ribonucleic acid fractions.**

The total amount of phosphorus in phospholipid fraction was determined by the method of Allen.[1] The amount of ribonucleic acid was determined by orcinol reaction and the mean phosphorus content of ribonucleic acid was estimated as 9%.

To avoid the adsorbed containation of ^{32}P, the inorganic phosphorus in phospholipid fraction was precipitated by addition of magnesium choloride. Thus, 96-99% of contamination was excluded. To extract contaminated inorganic ^{32}P,

the ribonucleic acid fraction was treated with isobutanol extraction.[2] This method eliminated about 85–95% of inorganic phosphate. The radioactivities were determined by a thin-mica windowed G-M counter. The specific activity and the relative specific activity were calculated by the following formula:

$$\text{specific activity} = \frac{\text{counts per minutes}}{\text{the amount of phospholipid-P or nucleic acid-P } (\gamma)}$$

$$\text{relative specific activity} = \frac{\text{(specific activity of "0-time" experiment)} - \text{(specific activity of sample)}}{\text{specific activity of incubation media or blood inorganic-P}}$$

Experimental Results

Table 1. Phosphorus metabolism of mammary tumor after 32 P-administration *in vivo*.

Case No.	Histological diagnosis	Age	Phospholipid-P γ/g	Relative sp. ac. of phospholipid %	Ribonucleic acid γ/g	Relative sp. ac. of ribonucleic acid %
16	Adenosis	44	44	1.59	1050	0.81
19	Adenosis	35	109	4.45	1750	1.82
20	Adenosis	43	127	5.66	1325	2.50
22	Adenosis	44	43	13.30	945	273.1
17	Fibrosis	39	76	2.90	1250	1.43
24	Carcinoma	53	133	4.31	1525	22.10
39*	Adenosis	34	133	37.2	1375	10.03
45*	Adenosis	28	153	2.82	2913	53.0
45*	Surrounding tissue	28	37	7.85	563	21.2

* Extirpated 24 hours after ^{32}P-injection.

Table 1 demonstrates the results obtained in ***in vivo*** experiments. As shown in Table 1, these data did not indicate any appreciable differences between normal and cancer tissues.

The influence of hormone was shown in Table 2.

Table 2. Influence of sex-hormone on phosphorus metabolism of mammary tumor after ^{32}P-injection.

Case No.	Histological diagnosis	Age	Phospholipid-P γ/g	Relative sp. ac. of phospholipid	Ribonucleic acid γ/g	Relative sp. ac. of ribonucleic acid	Hormone
35	Adenosis	36	164	0.29	875	1.12	Stanaprol
33	Fibrosis	41	40	9.42	1150	10.20	〃
34	Fibrosis	51	24	4.10	700	2.21	〃
31	Carcinoma	47	412	2.07	1875	6.31	Ovahormone

Here also, the administration of sex hormone did not give any appreciable effect on the phosphorus metabolism of mammary tumors.

In contrast to these data obtained by the *in vivo* experiments, those obtained by the *in vitro* experiments showed remarkable differences between the group of mastopathia and that of cancer. Table 3 demonstrates the results obtained by the *in vitro* experiments. The amounts of phospholipid in cancer tissue exceeded those in adenosis or fibroadenoma. The amounts of phospholipids-P of the latter group were 28-102γ per gramm of fresh tissue whereas the amounts of phospholipids in cancer were 114-231γ per gramm of fresh tissue except the case No. 43. However, as the relative specific activities of phospholipid did not increase in cancer, the biosynthesis of phospholipid seems not likely to increase by malignant change.

The amount of ribonucleic acid increased generally in cancer. But the most significant change in cancer was the increased relative specific activities of ribonucleic acid. Thus, it might be concluded that the biosynthesis of ribonucleic acid apparently increases in cancer tissue.

Table 3. Phosphorus metabolism of mammary tumor. (Experiments *in vitro*)

Case No.	Histological diagnosis	Age	Phospholipid-P γ/g	Relative sp. ac. of phospholipid %	Ribonucleic acid γ/g	Relative sp. ac. of ribonucleic acid %
5	Adenosis	39	65	0.001	—	—
7	〃	43	33	0.240	—	—
12	〃	44	52	0.847	—	—
29	〃	32	69	0.224	1350	0.564
32	〃	42	44	1.945	500	0.320
44	〃	42	28	0.232	562	0.109
50	〃	37	102	0.213	1813	0.356
9	Fibroadenoma	21	76	0.305	—	—
10	〃	38	56	0.001	—	—
11	〃	26	63	0.001	—	—
30	〃	43	49	0.995	580	0.146
48	Gynecomastia	22	74	0.145	625	0.438
58	〃	54	173	0.971	963	0.099
59	〃	54	300	0.343	788	0.310
8	Carcinoma	45	129	0.185	—	—
13	〃	57	114	0.164	—	—
14	〃	53	153	1.600	—	—
23	〃	74	131	0.906	1545	4.230
43	〃	95	69	0.142	188	2.416
46	〃	38	140	0.322	1425	2.192
47	〃	58	153	0.165	1688	0.519
49	〃	46	231	0.523	1788	2.443

Table 4. Influence of administration of sex hormone on the *in vitro* phosphorus metabolism of mammary tumor.

Case No.	Histological diagnosis	Age	Phospho-lipid-P γ/g	Relative sp. ac. of phospho-lipid	Ribo-nucleic acid γ/g	Relative sp. ac. of ribonucleic acid	Hormone
27	Adenosis	52	68	0.009	500	0.001	Stanaprol 50mg×8
38	〃	45	39	0.338	850	0.064	PHP 1g×10
41	〃	45	26	0.087	450	0.189	PHP 1g×5
42	〃	38	28	0.747	588	0.128	PHP 1g×10
25	Carcinoma	55	132	0.483	500	2.292	Stanaprol 50mg×7
26	〃	50	40	0.414	1820	0.336	Ovahormone 20,000u×30
28	〃	47	319	0.091	2000	0.049	Stanaprol 50mg×7
36	〃	38	133	0.143	1525	0.172	Stanaprol 50mg×9
40	〃	62	145	0.110	863	0.018	Ovahormone 20,000u×7
51	〃	40	320	0.111	1063	0.194	Stanaprol 50mg×10
54	〃	54	218	1.431	1125	3,578	Stanaprol 50mg×10

Table 5. The effect of 8-azaguanine on the *in vitro* phosphorus metabolism of mammary tumor.

Case No.	Histological diagnosis	Age	Dose of 8-azaguanine	Ribonucleic acid γ/g	Relative sp. ac. of ribonucleic acid	Difference
46	Carcinoma	38	—	1425	2.192	0
			10^{-3}M	973	1.543	−30%
			2×10^{-3}M	1288	1.275	−43%
47	Carcinoma	58	—	1688	0.156	0
			10^{-3}M	1563	0.087	−54%
			2×10^{-3}M	1063	0.186	−64%
51	Carcinoma with Stanaprol 50mg×10	40	—	1063	0.078	0
			10^{-3}M	850	0.066	−74%
			2×10^{-3}M	1340	0.0004	−95%
53	Carcinoma with Stanaprol 50mg×10		—	1525	0.225	0
			2×10^{-3}	1813	0.131	−42%

The effect of sex hormone administration prior to the surgical operation was

shown in Table 4. Although there were no remarkable changes in the phospholipid content of cancer, the relative specific activities of ribonucleic acid in cancer showed a dramatic decrease after the administration of male hormone. However, as seen in the Cases No. 25 and No. 54, when the age of patients were over fifty, the relative Specific activities of ribonucleic acid did not response to the administration of male hormone. In such aged cases (Cases No. 26 and No. 40) the administration of female hormone tended to decrease the relative specific activities of ribonucleic acid.

The effect of 8-azaguanine on the *in vitro* phosphorus metabolism of mammary tumor was demonstrated in Table 5.

The addition of 8-azaguanine tended to decrease the relative specific activity of ribonucleic acid as much as 35-75% of the conctrol. Furthermore, the reduction of the nucleic acid metabolism was remarkable when the male hormone was administered to the patients before the experiment.

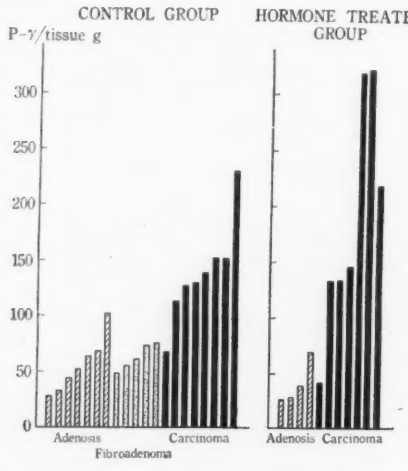

Chart 1. Relative specific activities of RNA in mammary tumors

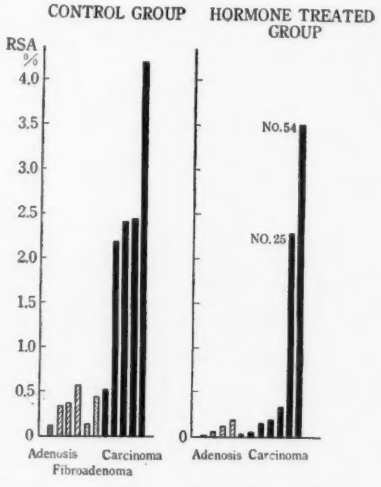

Chart 2. Phospholipid contents of mammary tumors

Discussion

According to the extensive work of Chaikoff *et. al.*,[3] the main site of the phospholipid synthesis in the human body is considered as the liver. In addition to this organ, the mammary carcinoma carry out also some parts of phospholipid synthesis since ^{32}P was incorporated into phospholipid fraction of the tumor.

However, there are several incompatible reports on the turnover rate of phospholipid in cancer tissue: Cornatzer,[5] Shalman[6] and Chaikoff[4] independently

reported higher turnover rate in cancer than in normal mammary tissue; on the other hand, Costello[7], Trew[8] and Albert[9] obtained fairy normal values like ours for phospholipid turnover.

The larger amount of phospholipid in cancer than normal tissue was reported by McEmen[10], Buelock[11] and Jowett[12]. The evidence of low turnover rate and high amount of phospholipid in cancer will suggest that a large amount of phospholipid was carried by the blood stream from the liver to the tumor before the surgical operation. There is good reason to support this view because Haven[13] observed high lipemia in rats bearing Walker-Carcidoma 256. The reason why the blood deposits phospholipid markedly in the tumor is till unexplained.

The informations concerning the content of ribonucleic acid in tumors are also controversial: Caspersson[14] and Price[15] observed higher amount of ribonucleic acid in hepatoma than in normal tissue, whereas Mills[16] reported lower amount of ribonucleic acid in hepatoma.

Although the amount of ribonucleic acid was not definitely increased in our data, the relative specific activity of this compound was higher in carcinoma than in mastopathia. In a case of adenosis (No. 29), we observed an abnormally higher relative specific activity of ribonucleic acid. In fact, the clinical observation demonstrated that this lesion was soon to undergo malignant changes. Thus, an observation of the high relative specific activity of ribonucleic acid will suggest a metabolic change toward cancer even when the histological observation has not yet indicated malignancy.

The remarkable changes were observed when sex hormones were administered prior to the surgical operation. The administration of male hormone to the relatively young carcinoma patients showed a marked decrease of the relative specific activities of ribonuclice acid. However, if the patients were relatively old (Cases No. 25 and 54) the administration of male hormone did not manifest any appreciable effect on the relative specific activity of ribonucleic acid. In fact, Haddow[17] suggested that the administration of female hormone would be rather favorable for the patients of mammary carcinoma after menopause.

The inhibitory effect of 8-azaguanine to the ribonucleic acid metabolism was established by Davidson *et al.*[18] Our experiment on 8-azaguanine showed also a remarkable decrease of the relative specific activity of ribonucleic acid in human mammary carcinoma.

Conclusion

Using ^{32}P as a tracer, the authors studied the metabolism of phospholipid and ribonucleic acid in human mammary tumors. Although there were no remarkable difference between mastopathia and cancer when ^{32}P was injected

intravenously, the *in vitro* administration of ^{32}P revealed several metabolic differences:

1) The phospholipids contents of mammary carcinoma were usually higher than those of adenosis or fibroadenoma.

2) The relative specific activities of ribonucleic acid were higher in cancer than in mastopathy.

3) The administration of sex hormone prior to the surgical removal of tissue decreased the relative specific activities of ribonucleic acid in cancer tissue.

4) The effect of 8-azaguanine *in vitro* showed a remarkable decrease of the relative specific activity of ribonucleic acid in cancer.

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要　旨

乳癌の核酸代謝の研究
—特に性ホルモンの影響について—

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要旨：　ヒトの乳腺腫瘍，特に乳腺症，乳癌などの推移に著者等は興味をもち，次のような実験を行った。すなわち ^{23}P をトレーサーとして，あるいは患者に静脈内注射を行った後に外科的に乳腺組織を剔出，あるいは剔出した組織を in vitro で ^{32}P とともに反応を行わせ，乳腺組織の燐脂質及びリボ核酸を抽出してその量と放射能を測定した。その結果，^{32}P を静脈内注射した例では一定の傾向は得られなかったが，in vitro で反応させた場合には次のような傾向がみられた。

(1)　乳腺症組織よりも乳癌組織の方に燐脂質量の多いこと。

(2)　乳腺症組織よりも乳癌組織の方がリボ核酸の相対比放射能が高いこと。

(3)　予め男性ホルモンを注射した患者の乳癌組織のリボ核酸の相対比放射能は低いこと。

(4)　50 才以後の患者には 男性ホルモンより女性ホルモンの投与の方が 乳癌組織のリボ核酸の相対比放射能を低めること。

(5)　8-アザグアニンは in vitro で乳癌組織のリボ核酸の相対比放射能を低めること。

以上の事実から著者等は次のように推論している。すなわち

(1)　乳癌組織自体の燐脂質の 相対比放射能はさして 高くしないところから，乳癌に燐脂質量の多いのは肝において合成され血流によって運ばれる燐脂質が乳癌組織では乳腺症組織にくらべ特によく沈着するのであろう。

(2)　リボ核酸の相対比放射能の高低はおそらく 蛋白合成の度に 平行関係のあることを 示唆するから，乳癌組織では乳腺症組織にくらべ蛋白合成が盛であると考えられ，かつこの代謝は性ホルモンのバランスによって影響を受けているものと思われる。

[GANN, 48, 199~203; June, 1957]

THE INFLUENCE OF ESTROGEN ON THE RIBONUCLEIC ACID METABOLISM OF RAT LIVER

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INTRODUCTION

In the course of the experiments on the phosphorus metabolism of human mammary tumor, the authors found that the sex hormone administration to the patient prior to the surgical removal of mammary tumor was followed by a deep decrease of the ribonucleic acid metabolism of mammary tumors. Thus the question arose whether the sex hormone has some influence on the ribonucleic acid metabolism of other organs. In order to observe the effect of estrogen on the liver ribonucleic acid metabolism, the authors performed the following experiment.

EXPERIMENTAL

Young female rats weighing about 100g were divided into four groups: (I) Normal group; (II) Bilaterally ovariectomized group; (III) Normal group with the administration of estrogen (200–250 IU of Estradiol benzoate "Teikokuzoki" 10 doses, 1–2 days' interval); (IV) Ovariectomized group with the administration of estrogen.

Table 1. Composition of the incubation medium for homogenate.

32p (as $Na_2H\ ^{32}PO_4$) 50 μc	2.0 ml
0.5 M KCl	2.0 ml
0.1 M $MgCl_2$	0.5 ml
4×10^{-4} M Cytochrome c	0.5 ml
0.01 M Adenosine triphosphate-K salt	1.5 ml
0.03 M α-Ketoglutarate-K salt	1.5 ml
0.154 M KCl	2.0 ml
0.1 M Phosphate buffer (pH 7.4)	1.0 ml
20% Liver homogenate with isotonic KCl	5.0 ml

The incubation time was 60 minutes for all cases.
The experimental technique was quite the same as the previous report.[1)]

Soon after the animal was sacrified by decapitation the livers were removed; and the tissue was sliced or homogenated. The incubation medium containing ^{32}P for slice was the same to that used in previous experiment.[1)]

The composition of the incubation medium for homogenate was indicated in Table I.

Results

The experimental results with liver slice were shown in Table II, and those of liver homogenate in Table III.

Table 2. Experiments with liver slices.

Exp. No.	Phospholipid-P γ/wet tissue g				Relative sp. ac. of phospholipid				Ribonucleic acid γ/wet tissue g				Relative sp. ac. of ribonucleic acid			
	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
1	565	535	584	584	0.32	0.36	0.46	0.49	3250	3050	4590	3850	0.05	0.07	0.06	0.14
2	678	469	794	669	0.26	0.52	0.30	0.41	3120	3925	3980	5035	0.11	0.07	0.05	0.12
3	581	517	581	518	0.23	0.26	0.13	0.27	3270	3100	3495	3900	0.19	0.20	0.18	0.21
4	653	675	645	615	0.25	0.46	0.55	0.59	4295	4265	4810	5000	0.35	0.31	0.31	0.59
5	690	676	606	578	0.42	0.26	0.34	0.45	3760	4235	4335	4055	0.37	0.35	0.30	0.56
6	684	846	578	815	0.68	0.60	0.83	0.71	5660	5710	3750	5470	0.18	0.24	0.33	0.35
7	796	638	638	538	0.69	0.69	0.66	0.53	4335	4235	3700	3535	0.14	0.06	0.30	0.24
Mean	650	622	632	617	0.42	0.45	0.47	0.49	3957	4029	4094	4406	0.20	0.19	0.22	0.32

Table 3. Experiments with liver homogenates.

Exp. No.	Phospholipid-P γ/wet tissue g				Relative sp. ac. of phospholipid				Ribonucleic acid γ/wet tissue g				Relative sp. ac. of ribonucleic acid			
	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
1	503	304	275	238	0.71	0.06	0.16	0.64	3065	2010	3295	3785	0.03	0.04	0.02	0.03
2	495	486	570	540	0.46	0.19	0.19	0.67	3370	3455	3210	3570	0.13	0.06	0.01	0.11
3	581	450	341	375	0.38	0.83	0.80	0.94	3265	3490	2890	2845	0.01	0.32	0.23	0.10
4	571	610	604	529	0.62	0.89	0.91	0.75	4015	5200	5200	4245	0.55	0.51	0.51	0.51
5	538	381	469	544	0.08	0.18	0.43	0.53	3570	2870	3980	3190	0.45	0.23	0.48	0.55
6	673	595	677	514	0.96	1.73	1.34	1.61	5100	4845	4360	4845	0.04	0.18	0.06	0.05
7	613	671	644	598	1.07	1.04	0.81	0.92	3290	3570	3161	3927	0.29	0.15	0.15	0.12
Mean	568	500	511	477	0.56	0.70	0.66	0.87	3668	3763	3712	3772	0.21	0.21	0.21	0.21

Although there were several seasonal fluctuations of values between each experiment, the following tendency will be found after the comparison of values between four groups in each experiment:

(1) Though the bilateral ovariectomy itself did not give any appreciable influence on the ribonucleic acid metabolism, there occurred a marked increase in the relative specific activity of ribonucleic acid by the administration of estrogen to ovariectomised rats. (1.5–4.2 times increase in the group IV as that in the group II). The difference was significant at the 5 per cent level. On the other hand,

there was no significant increase in the relative specific activity of ribonucleic acid by the administration of estrogen among normal rats.

(2) Similar tendencies were observed in the increase rate of the relative specific activity of phospholipid. However it was not statistically approved.

(3) In the series of experiments with homogenate, there were no difference among the groups in phospholipid and ribonucleic acid metabolism.

Discussion

Since Mandel[2] reported in 1947 an increased concentration of blood nucleic acid after the injection of estrogen, there were several observations concerning the effect of estrogen on the metabolism of liver nucleic acid. Chapman *et al.*[3] observed in 1949 an increase of the liver weight and liver protein after the administration of estrogen to immature pullets. Moreover, they reported again in 1951[4] that the total number of liver cells and the amount of deoxyribonucleic acid per liver increased after the administration of estrogen, whereas the amount of ribonucleic acid did not increase at all. In 1952, Phillips *et al.*[5] found that the ratio of ribonucleic acid to protein in rat liver increased remarkably after the injection of estradiol. Furthermore, they obtained evidence that the administration of estradiol was not associated with the formation of the larger amount of deoxyribonucleic acid in chicken liver.[6]

No change in the amount of ribonucleic acid in liver cytoplasma was observed by Reid[7] in 1956 after the ovariectomy of rats. Baum *et al.*[8] found that the administration of diethyl stilbesterol did not involve any change in the amount of lipid in chicken liver.

Our data concerning the constancy of the amount of liver phospholipid and ribonucleic acid after the ovariectomy or administration of estrogen are in good accordance with the reports of Chapman, Reid or Baum. An increase of the relative specific activity of ribonucleic acid might suggest some similarity with regenerating livers which Chapman observed, e. g., an increase of cell number as well as deoxyribonucleic acid showed also a resemblance to regenerating livers.

The fact that the effect of estrogen on ribonucleic acid metabolism became manifest only after the ovariectomy will suggest an existence of some unknown hypophyseal factor which may respond to the ovareal estrogen and control the secretion of the latter in reverse order. After the ovariectomy this hypophyseal factor would not be yielded; consequently the administration of estrogen would not be controled by this factor. In fact, Campbell *et al.*[9] reported in 1953, that the amount of liver ribonucleic acid did not change by the ovariectomy nor by the administration of estrogen to the normal rats, whereas the amount of ribonucleic acid per gramm of protein increased considerably by the injection of estradiol to

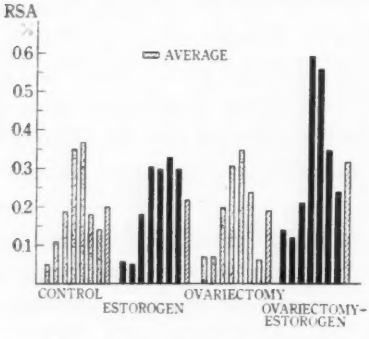
the hypophysectomized rats.

The experimental results with homogenate may suggest that the effect of hormone will act only within the cellular level, so the destruction of cells could not get any influence of hormone.

Conclusion

In order to know the estrogenic influence on the metabolism of liver phospholipids and ribonucleic acid, the authors carried out a series of the following experiments; 28 female rats were divided into 4 groups, namely, control group, ovariectomized group, control group with the administration of estrogenic hormone and ovariectomized group with the administration of estrogenic hormone.

The livers were removed and sliced tissues were incubated in vitro with ^{32}P. The incorporation rates of ^{32}P into phospholipids and ribonucleic acids were calculated.



Chapt. 1. Estorogenic Influence on RNA Metabolism of RAT Liver Slice

The influence of estrogen was remarkable in the increased rate of ^{32}P-incorporation into ribonucleic acids when the comparison was made between the ovariectomized and those with the administration of estrogen. The lipids and ribonucleic acid contents did not show any difference among the four groups.

Furthermore, it was demonstrated that the effect of estrogen was lost by the homogenation of tissue.

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要　旨

ダイコクネズミの肝のリボ核酸代謝に及ぼす エストロゲンの影響

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要旨：　乳癌患者に男性ホルモンを投与すると乳癌組織のリボ核酸代謝が低下する事実をみとめたので，次に性ホルモンが Target organ 以外の臓器の代謝にも影響を及ぼすか否かをみるため次のような実験を行った。

すなわち体重 100 g 前後の雌のダイコクネズミ 4 匹づつ 7 組を飼養し，各組毎を次のような区分に分けた。(1)　対照の正常ネズミ，(2)　両側の卵巣剔除を行ったネズミ，(3)　正常のものにエストロゲンを投与したネズミ，(4)　卵巣剔出を行った後にエストロゲンを投与したネズミ。

これらのネズミを断頭して肝をとり出し，そのスライスまたはホモジネートと ^{32}P を in vitro で反応せしめ，後に燐脂質分劃及びリボ核酸分劃を抽出，その量と放射能を測定した。その結果，スライスによる実験では，卵巣を剔出したネズミにエストロゲンを与えると肝のリボ核酸代謝が昂進することが明らかになった。このことはホモジネートでは統計的に明らかではない。

これらの事実から性ホルモンの Target organ でない肝でも性ホルモンはそのリボ核酸代謝に影響を及ぼすこと，及びその影響は細胞のレベルまででホモジネーションによって消失することが明らかになった。さらにこれらのエストロゲンの影響が卵巣剔出によって明瞭になる事実は何か卵巣からの刺激によって脳下垂体から或因子が分泌され，これが逆に卵巣のエストロゲン分泌を抑制しているのではないかという推察を与える論拠となるものである。

[GANN, 48, 205~217; June, 1957]

ON THE ANTI-CANCEROUS EFFECT OF P-PHENYLENE-DIPHOSPHORIC ACID TETRAETHYLENIMID (O, O'-P-PHENYLENE N, N', N'', N'''-TETRA-ETHYLENE-TETRAMIDODIPHOSPHATE)

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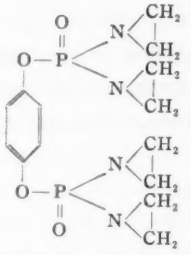
Of chemotherapeutic drugs for cancer, N, N', N''-triethylenemelamine (TEM)[1] of the ethylenimine group and N, N', N''-triethylenephosphoramide (TEPA)[2,3] N, N', N''-triethylene-thio-phosphoramide (Thio-TEPA, TSPA)[4] and other similar phosphatic compounds are regarded today as quite promising. Also, the anticancerous effect of the quinone derivatives, 2, 5-diethylenimino-1, 4-benzoquinone and 3, 6-alcoxy-2, 5-diethylenimino-1, 4-benzoquinone, is acknowledged by Domagk,[5,6,7] et al. Among the various ethyleneimide compounds synthetized by Sunagawa and others,[8] p-phenylenediphosphoric acid tetraethyleneimid (O, O'-p-phenylene N, N', N'', N'''-tetraethylene-tetramidodiphosphate) has been found to be particularly effective in destroying Ehrlich ascites carcinoma of both ascitic and subcutaneous tumor types and transplanted mammary cancer of C3H and A mice. The experimental results are given below.[9]



p-Phenylenediphosphoric acid tetraethylenimid
(O, O'-p-phenylene N, N', N'', N'''-tetraethylene-tetramidodiphosphate)

Conforming to the conclusion of the match test with the Screening Method,[10] which was presented at the American Cancer Society, the experiments were carried out principally with transplanted cancer in animals. The agents tested were first selected by screening with Ehrlich ascites carcinoma and then administered to mice with transplanted mammary cancer for further confirmation of their efficacy. Increase in body weight owing to accumulation of ascites, growth of tumor and prolongation of life were observed in evaluating the results; furthermore, for reference, the effect on the mitosis of the cells of Ehrlich ascites

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carcinoma was examined microscopically.

1. Chemical agent.

The chemical compound p-phenylenediphosphoric acid tetraethylenimide synthetized by Sunagawa and others was used. At first, as comparison with other chemical agents was considered, a suspension of the drug in 0.5% CMC physiologic saline[11] was used. This suspension was preserved at 0°±1° C immediately after preparation and used in the experiments from the following day. Five hundred units of penicillin and streptomycin were always added to each 1.0 cc of the suspension before use. As this suspension proved to be remarkably effective, this drug in a purified form was dissolved in physiologic saline just before injection and the results were compared with those of the suspension. Although easily soluble in physiologic saline, this substance when exposed to room temperature, light or moisture gradually becomes insoluble. The solution was therefore prepared only when needed just before injection and the drug was preserved in a desiccator under reduced pressure at 0°±1° C.

2. Experimental animals.

SM and ddN mice (Central Laboratories of Experimental Animals, Tokyo, Japan), 4 to 5 weeks old, ranging in weight from 18 to 22 gm. were used for the experiment with Ehrlich ascites carcinoma, after the method of Tadashi Yamamoto,[12] et al. Transplanted mammary cancer was produced by transplanting a spontaneous cancerous growth from a C3H mouse (supplied by Prof. Mori of Showa Medical College) to the first filial generation[19] between SM and C3H mice ($SM \times C3H/F_1$) four weeks after birth.

A spontaneous cancerous growth from an A mouse (supplied by the National Institute of Genetics) was transplanted to mice, 3 to 4 weeks old, of the first filial generation[19] between ddN and A mice ($ddN \times A/F_1$) to produce the transplanted mammary cancer.

3. Experimental methods.

A. Experiments using Ehrlich ascites carcinoma.

a) In vitro test.

Ascites from a mouse 8 days after inoculation was aspirated slowly with a syringe; 0.5 cc of this fluid was poured into a test tube, to which 0.5 cc of normal saline and 0.5 cc of a 3% or 6% CMC suspension of p-phenylenediphosphoric acid tetraethyleneimid were added. After being kept at 38° C for 60 minutes, 0.1 cc of this mixture (containing approximately 15,000,000 tumor cells) was transplanted intraperitoneally into each of six SM mice. For controls, a solution containing 0.5 cc of ascites, 0.5 cc of normal saline and 0.5 cc of the CMC solution was used. The increases in body weight due to accumulation of ascites in the two groups were compared. This method is based on that of Fukuoka and others.[13, 14]

b) In vivo test.[15, 16, 17]

Approximately 0.05 cc of the ascites fluid (containing 1,000,000 tumor cells), aspirated 7 days after inoculation was diluted with a fixed amount of normal saline and injected into the peritoneal cavity of ddN mice. Twenty-four hours after implantation of the tumor cells, the test agent dissolved in 0.20 cc of CMC solution or normal saline was injected into the peritoneal cavity of mice once daily for 6 consecutive days. Each group of 4 to 15 mice was kept under identical supervision and conditions in a metal breeding box, fed with "Oriental solid food" and supplied with sufficient water from a bottle specially designed for this purpose. The average weight of the treated group was compared with that of the controls, and the survival time was noted to calculate the length of prolongation of life. Observation was thus continued for 35 days. Normal saline or CMC solution was injected into the mice in the control group and for comparison 6-mercaptopurine was also used.

c) Effect on the mitosis of ascites tumor cells of Ehrlich carcinoma.

Each group of 3 mice 8 days after transplantation of tumor cells received intraperitoneal injections of 90 mg/kg of p-phenylene-diphosphoric acid tetraethylenimid (CMC suspension) daily for 3 consecutive days. One group of mice was sacrificed daily after 24 hours following the injection and the ascites fluid collected. A portion of this fluid was stained with aceto-gentianaviolet and examined microscopically. Five thousand tumor cells were counted to determine the ratio of cells at the rest period and those at the division period. CMC solution was injected in the same manner into the mice in the control group.

B. Effect on solid carcinoma.

a) Effect on subcutaneous tumors of Ehrlich ascites carcinoma.

Subcutaneous tumors were induced by injecting hypodermically ascites fluid containing 16,000,000 tumor cells diluted with normal saline to SM mice. To each group of 5 mice, beginning from the 3rd day after inoculation, 40 mg/kg of a CMC suspension of p-phenylenediphosphoric acid tetraethylenimid was injected subcutaneously once a day around the tumor for 7 consecutive days and the inhibiting effect on the growth of tumors was observed.

b) Effect on mammary cancer of C3H mice.[18]

Groups of 5 to 7 mice over 4 weeks of age were picked from the first filial generation[19] of female SM mice mated to C3H mice ($SM \times C3H/F_1$) for the experiment. Tumor pieces from spontaneous cancer were implanted subcutaneously in the back of each mouse. Three groups of mice received hypodermic injections of the drug around the tumors from 24 hours, 3 days and 7 days after the transplants respectively. Daily doses of 20 to 60 mg/kg of the drug were injected once every day or every other day 7 or 20 times. The control group received an equal amount

of CMC solution. The effect of the drug on the growth of tumors was observed.

c) Effect on mammary cancer of A mice.

To the first offsprings[19] of a female ddN mouse mated to an A mouse (ddN× A/F_1), 3 to 4 weeks after birth, tumor pieces from spontaneous cancer were transplanted subcutaneously on the back. Eight days after transplantation, mice with tumors of an equal size (3×3×3 mm) were selected, and a daily dose of 40 mg/kg was injected hypodermically around the tumors every other day for 12 to 6 times. CMC solution was injected in the control group and the action of the drug on the growth of tumors was observed.

4. Experimental results.

Lethal doses were determined with mice in the same condition as those that were used in the experiments for evaluating the anticancerous effects of the drug.

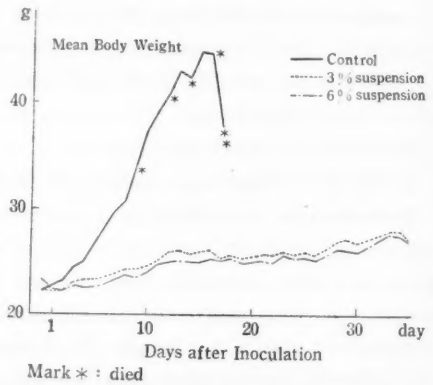
	Intraperitoneal mg/kg	Hypodermical mg/kg	Intravenous mg/kg
In CMC solution	195-221	226-250	—
In physiologic saline	177-217	202-224	190-215

A. Experiments using Ehrlich ascites carcinoma (Fig. 1).

a) In vitro test.

As shown in Fig. 1, while ascites cancer grew normally in the control group, no increase of body weight owing to accumulation of ascites in the treated group was observed, nor did any deaths due to ascites cancer occur in this group.

Fig. 1. In vitro test.



Mark * : died

b) In vivo test.

(1) Administration of a suspension of the drug in CMC solution.

When a 10 mg/kg dosage was administered, although the increase in body weight was almost parallel to that of the controls, the survival rate of 22.41 ±4.48 days on an average surpassed the average of 14.8±0.64 days for the controls.

With a 20 mg/kg dose, life was prolonged for 35 days in one of 5 mice, while 2 died on the 31st days and one each on the 19th and 29th days. The curve of the average body weight of the treated group is lower than that of the control group which evidently indicates the cytostatic action on ascites cancer.

When the dose was 40 mg/kg, 11 of 15 mice survived for more than 35 days. Two died on the 18th day, one on the 22nd and another on the 28th. The curve

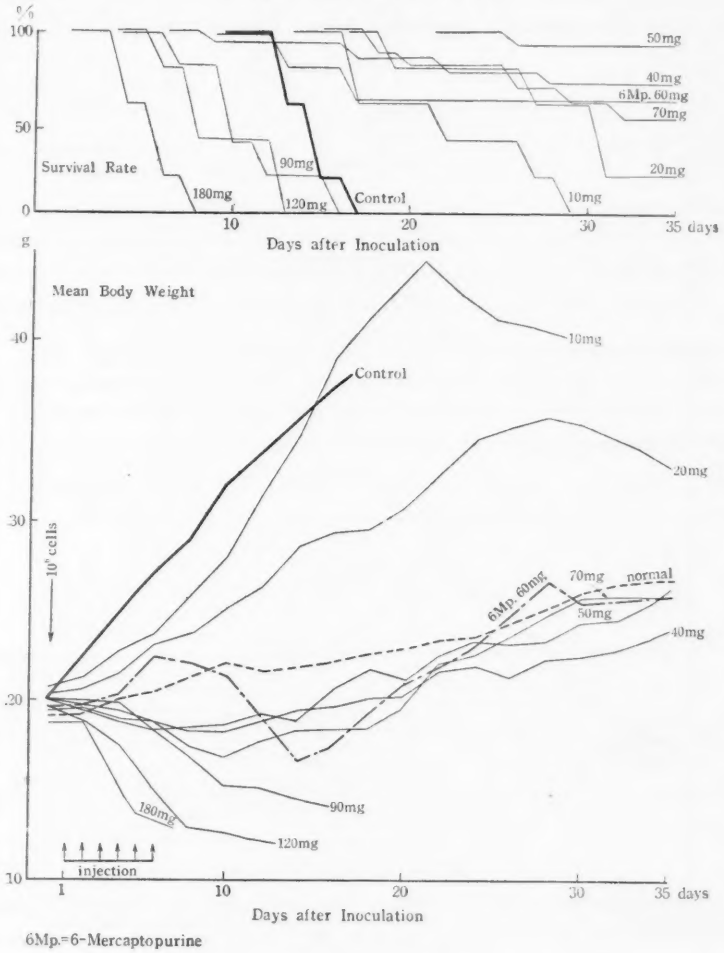
of average weight increase was approximately equivalent to that of normal growth.

When the dose was 50 mg/kg, prolongation of life for more than 35 days was observed in 14 of 15 mice, one mouse dying on the 25th day. The average weight increase was almost identical with that of normal growth.

A dosage of 70 mg/kg prolonged the lives of 8 of 15 mice for more than 35 days, and the average weight increase was almost normal.

When 90 mg/kg was injected the average days of survival was 11.0±3.4 days which was less than that of the control group. This is probably due to the toxic effects of the drug.

Fig. 2. In vivo test (1)



6Mp.=6-Mercaptopurine

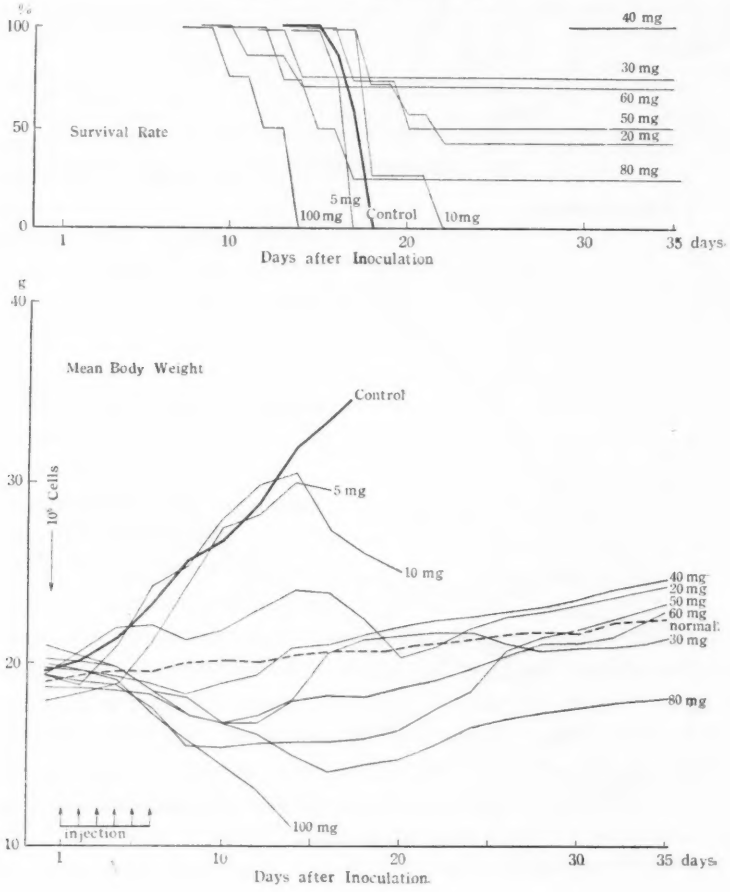
The average days of survival with dosages of 120 mg/kg and 180 mg/kg were 7.0±3 and 4.8±1.5 respectively. Both these groups presented severe toxic reactions to the drug.

When 6-mercaptopurine in dosage of 60 mg/kg was administered, the average weight curve was approximately similar to that obtained when doses of 40 mg 50 mg, and 70 mg per kilo gram of body weight of p-phenylene diphosphoric acid tetraethylenemid were injected, and 3 of 5 mice survived for more than 35 days. One died on the 13th day and another on the 17 day.

(2) Administration of the drug dissolved in normal saline (Fig. 3).

Approximately similar results were obtained as in the previous experiment when the drug was used in a suspended form. Each of the groups that received

Fig. 3. In vivo test (2)



20 mg, 40 mg, and 60 mg per kilo gram of body weight of the drug consisted of 7 mice including the control group, and the other groups of 4 mice each.

c) Effect on the mitosis of cells of Ehrlich ascites carcinoma.

Compared to the control group, there was a decrease in the number of mitotic cells in the treated group.

Table 1. Effect on the Mitosis of cells of Ehrlich ascites carcinoma.

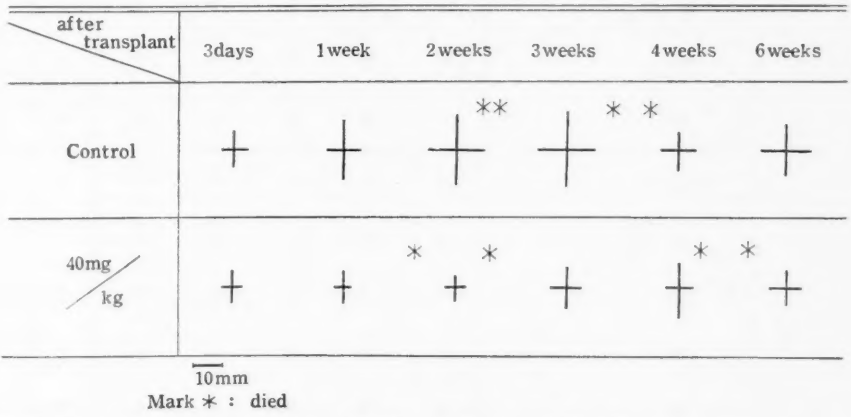
day		Mitosis / 5,000cells	Mitosis / Resting	Prophase / Mitosis	Metaphase / Mitosis	Anaphase / Mitosis	Telophase / Mitosis
1 st	Control	123 cells	2.46%	23.7%	43.2%	13.1%	19.0%
	Experiment	32	0.64	33.6	54.9	2.5	9.0
2 nd	Control	91	1.82	27.7	44.2	9.5	19.6
	Experiment	32	0.64	40.6	53.1	3.4	3.9
3 rd	Control	92	1.84	25.2	40.4	16.8	17.6
	Experiment	20	0.40	41.7	51.7	5.0	1.6

B. Effect on solid carcinoma (Fig. 4).

a) Effect on subcutaneous tumors of Ehrlich ascites carcinoma.

No conspicuous effect was observed with hypodermic injection of a CMC suspension around tumor.

Fig 4. Effect on subcutaneous tumor type of Ehrlich ascites carcinoma.
Injection is initiated 3 days after implant, once every day 7 times.
Measurement of long axis and short axis of tumor are represented mark +.



b) Effect on mammary cancer of C3H strain mice (Fig. 5).

Sugiura's method[20] of measuring and comparing the average diameter of the tumors with that of the controls was used in evaluating the effects of the drug.

− No effect; the average diameter was 3/4 or more the average diameter of the control tumors.

± Slight inhibition; the average diameter was 1/2 to 3/4 the average diameter of the control tumors.

Fig.5. Effect on Mammary cancer of C3H strain mice.

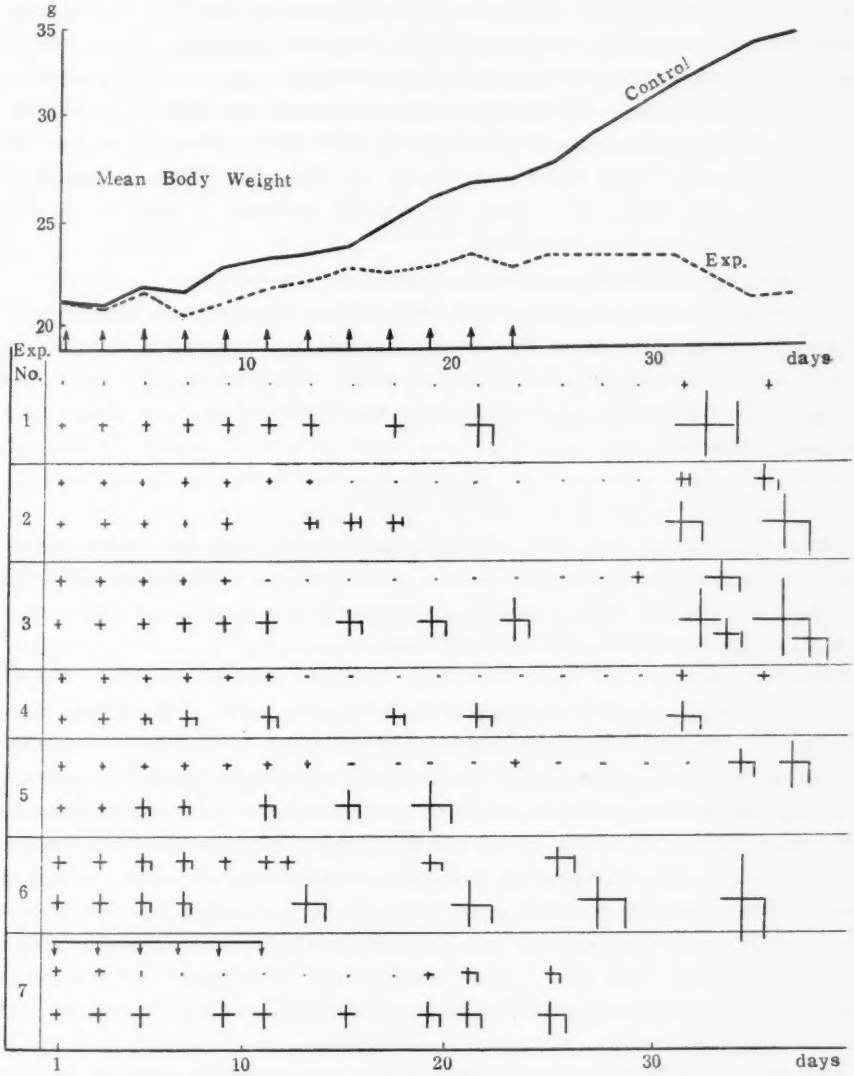
Class 1. Injection is initiated 24 hours after implant, once every other day 7 times.
〃 2. Injection is initiated 3 days after implant, once every other day 7 times.
〃 3. Injection is initiated 7 days after implant, once every day 7 times.
〃 4. Injetion initiated 7 days after implant, once every other day 20 times.
Measurement of long axis and short axis of tumor are represented mark +.

Class	Daily doses \ Weeks after transplant	1	2	3	4	5	Evaluating
1	Control	+	+	+	+	+	−
	40 mg/kg	+	+	+	+	+	+
	60	+	+	+	+	+	++
2	Control	+	+	+	+	+	−
	40 mg/kg	+	+	+	+	+	+
	60	+	+	+	+	+	++
3	Control	+	+	+	+	+	−
	40 mg/kg	+	+	+	+	+	±
	50	+	+	+	+	+	+
	60	+	+	+	+	+	+
4	Control	+	+	+	+	+	−
	20 mg/kg	+	+	+	+	+	−
	30	+	+	+	+	+	±
	40	+	+	+	+	+	±
	50	+	+	+	+	+	++
	60	+	+	+	+	+	++

10 mm

+ Moderate inhibition; the average diameter was 1/4 to 1/2 the average diameter of the control tumors.

Fig. 6. Effect on Mammary cancer of A strain mice.



Measurement of long axis, short axis and perpendicular axis of tumor are represented by mark +. The day of treatment is shown by ↑ ↓ mark. The upper is the treated. The under is the control.

$\dagger$ Marked inhibition; the average diameter was 1/4 or less that of the control tumors.

$\ddagger$ Complete inhibition or destruction of tumors.

With institution of treatment, retardation of growth or diminution in size of the tumors was observed. The size of the tumors in the fifth week, when treatment was started 24 hours or 3 days after transplantation and a total of 7 injections was given (on alternate days) was + and $\dagger$ in mice receiving doses of 40 mg/kg and 60 mg/kg respectively. When treatment was started on the 7 th day and administered daily for 7 days, the size was $\pm$ when 40 mg/kg was administered and + when 50 mg or 60 mg/kg was injected. When 20 injections were given once every other day, the tumors decreases in size to $\pm$ with 40 mg/kg and $\dagger$ with 50 or 60 mg/kg.

C. Effect on mammary cancer of A strain mice (Fig. 6).

Mice with tumors approximately 3×3×3 mm in size were treated. Six injections of 40 mg/kg given every other day greatly reduced the size of the tumors and they could hardly be palpated from the skin. However, about a week after termination of treatment, regrowth of the tumors occurred and they could again be easily palpated.

Discussion

The ascites cancer cells are generally used for the first stage in screening chemotherapeutic agents for the cure of cancer, not only because it makes the selection of hosts of inbred strains unnecessary, but also because the exact amount of implanted cells can be determined.

However, Sugiura[21] of Sloan Kettering Institute pointed out that Ehrlich ascites carcinoma cells are susceptible to substances other than anticancerous agents; for instance even weak alcohol has an effect on the cells of ascites carcinoma, and be emphasized the importance of using solid cancer for the tests.

In our studies with p-phenylenediphoric acid tetraethylenimid, we first observed the remarkably potent action of this chemical agent in restraining the increase in body weight and prolonging the lives of mice with Ehrlich ascites carcinoma and in succeeding experiments with C3H and A mice, curative effects were demonstrated with the same dosage as used in the experiments with Ehrlich ascites carcinoma. The solid cancer used for the experiments was induced in the first hybrid generation of SM×C3H/F_1 or ddN×A/F_2 stocks by transplanting spontaneous cancer cells.

As there is a difference between spontaneous and implanted cancers, the use of spontaneous cancer is most desirable and this is being planned for our future investigations, but breeding hosts of inbred strains is difficult and it is not easy

to maintain and prepare suitable conditions for the growth of spontaneous cancer.

Though the anti-cancerous effects of this drug in animals is yet to be verified in human beings, our experiments with implanted cancer have shown that of the ethyleneimine compounds, p-phenylenediphosphoric acid tetraethyleneimid is of promising value as a chemotherapeutic agent for cancer together with TEM,[1] TEPA,[2,3] TSPA,[4] 2,5-diethyleneimino-1,4-benzoquinone and 3,6-alcoxy-2,5-diethyleneimino-1,4-benzoquinone.[5,6,7] Further investigation with a broader spectrum of animal cancers and careful study of its effect on cancer in humans are required.

Conclusion

Biological experiments in regards to the anti-cancerous effects of p-phenylenediphosphoric acid tetraethyleneimid (O,O'-p-phenylene-N, N', N'', N'''-tetraethylenetetramidodiphosphate) were conducted, and the following results were obtained.

1. Lethal dose. The LD_{50} of this drug in mice in the same condition as those that were used in the investigation of anti-cancerous effects was 195-221 mg per kg when injected intraperitoneally and 226-250 mg per kg with hypodermic injections when the drug was suspended in CMC solution. When dissolved in physiologic saline the LD_{50} were 177-217 mg/kg, 202-224 mg/kg and 190-215 mg/kg when injected intraperitoneally subcutaneously and intravenously respectively.

2. ***In vitro*** tests with this drug were carried out with Ehrlich ascites carcinoma cells according to the method of Fukuoka and others. No growth of ascites cancer cells was observed in mice inoculated with the treated ascites fluid.

3. ***In vivo*** tests with Ehrlich ascites carcinoma were performed with intraperitoneal injections of the drug given once daily for 6 consecutive days after 24 hours following transplantation of 10^6 tumor cells. Dosage of 30 mg to 60 mg/kg was observed to be the most effective; with this dosage no accumulation of ascites was noted and the effect on prolonging life was also conspicuous. With 20 mg/kg dosage a slight effect was observed as compared with the controls, but no effect was obtained with 10 mg/kg. When 90 mg/kg or more was administered, death of the mice occurred earlier than in the controls due to the toxicity of the drug.

4. Decrease in mitosis of the tumor cells was observed when the drug was injected into the peritoneal cavity of mice with Ehrlich ascites carcinoma.

5. Subcutaneous injection around the tumors of Ehrlich ascites carcinoma produced no conspicuous effects.

6. Implanted mammary cancer to the first hybrid generation was used to

observe the effects on solid cancer. To produce mammary cancer in C3H mice, spontaneous cancer cells were transplanted to mice from the first litter of a female SM mouse mated to a C3H mouse. Retardation of growth of tumor was used as criteria in judging the effects of the drug. Similarly, for mammary cancer in A mice, cancer cells were transplanted to ddN×A/F_1 mice. In both groups the drug when injected subcutaneously around the tumor inhibited the growth of the tumor.

The authors wish to express their deepest gratitude to Drs. Kozo Hayashi and Kiyoshi Tsuchikawa of the National Institute of Genetics and Prof. Kazuo Mori of Showa Medical College for their advice. We are also greatly indebted to Messrs. Matsumi Fujii and Toru Akiba of this laboratory who were in charge of the breeding of experimental animals.

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要　旨

p-Phenylenediphosphoric acid tetraethyleneimid (O, O′-*p*-Phenylene N, N′, N″, N‴-tetraethylene-tetramidodiphosphate) の制癌作用について

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(高峰研究所)

砂川等が新しく合成した ***p*-Phenylenediphosphoric acid tetraethyleneimid (O, O′-*p*-Phenylene N, N′, N″, N‴-tetraethylene-tetramidodiphosphate)** は Ehrlich 腹水癌腹水型，皮下腫瘍型，C3H 系及び A 系ハツカネズミ乳癌（第一代雑種への移植癌）に対し制癌作用を示した。

1) LD_{50} (マウス)

腹腔内注射 177～217 mg/kg，皮下注射 202～224 mg/kg，静脈注射 190～215 mg/kg.

2) 薬剤を試験管内で Ehrlich 腹水癌腹水に作用させたところ，該腹水を接種したマウスは全く腹水癌の発生をみなかった。

3) 毎日1回6日間連続腹腔内注射で Ehrlich 腹水癌の発生を抑制した。30 mg～60mg/kg が有効量とみとめられる。

4) Ehrlich 腹水癌細胞の有糸分裂を抑制した。

5) Ehrlich 腹水癌皮下腫瘍周囲に皮下注射したが，いちじるしい効果はみとめなかった。

6) C3H 系マウスに自然発生した乳癌を SM×C3H/F_1 に移植し，また A 系マウスに自然発生した乳癌を ddN×A/F_1 に移植して，腫瘍周囲に皮下注射したところ，結節癌の増大抑制をみとみた。

[GANN, 48, 219~225 : June, 1957]

GEOGRAPHICAL OBSERVATION ON CANCER MORTALITY BY SELECTED SITES ON THE BASIS OF STANDARDISED DEATH RATE

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For comparative study of the geographical distribution of cancer, it is best suited to the purpose to use statistics on morbidity rate, but since at present there are not many regions in the world for which such statistics are available, and as the second best, it may not be insignificant to some extent to rely upon mortality statistics in making interregional comparisons. In attempting such comparisons, we must bear in mind that the age distribution of the population is not uniform by different regions, and therefoae it is desirable to compare the mortality from cancer on the basis of the standardised death rates in stead of the crude death rates.

1. Sources and Calculating Method.

The authors have applied to the central offices of vital statistics of 23 countries for the data on the mortality by age for cancer of all sites, and of the selected sites including stomach, esophagus, lung, breast, uterus, ovary, and prostate in the respective countries. On the basis of the data thus informed, the standardised death rates were computed. For the computation, the sex and age distribution of the Japanese population in 1953 was used as standard. The names and the respective years for which we made our adjustment are listed in the following:

1952-1954	Canada, Israel, Japan, Austria, Denmaak, Finland, France, Ireland, Norway, Netherlands, Portugal, England and Wales, Scotland, Northern Ireland, Australia, and New Zealand
1951-1953	Union of South Africa, U. S. A. (White and nonwhite populations), Italy, Sweden, and Switzerland
1952, 1953	German Federal Republic
1953	Chile
1954	Belgium

2. Comparison of Standardised Death Rates for Cadcers of All Sites

The subject death rates are shown in Table. Among males, the rate is the highest in Finland (134.4 per 100,000 male population in standardised rate), followed by Scotland (130.0), Austria (124.6), England and Wales (120.8), Switzerland (120.7) and so forth, while it is lowest in Portugal (58.9), Israel (83.3, Jewish

population only), Sweden (83.4), Italy (87.5) and Japan (88.1) also standing low in the list. Japan stands in the 21 st place in the descending order among the 25 countries listed (the statistics of U. S. A. being available in breakdown for whites and nonwhites, it has been counted as two items in the list). The rate of Finland, the highest, is about 2.3-folds that of Portugal, the lowest.

Among the female population, the highest rate is shown by Denmark (119.1), Chile (112.4), Austria (111.1), German Federal Republic (109.4), Scotland (107.3) and so forth following the lead, while the lowest rates are shown in Portugal (58.0), Japan (77.9), Italy (80.7), Australia (86.7) and France (87.6). Here, Japan shows the second lowest rate next to Denmark among the 25 countries. The highest rate shown by Denmark is about twice as high as that of Portugal, the lowest. It is noteworthy that Portugal has the lowest rates, both in male and female populations.

3. Comparison of Standardised Death Rates for Stomach Cancer

As shown in Table, the standardised death rate among males is highest in Japan (50.1), Chile (48.1) and Findland (46.4) showing particularly high rates next to Japan. Then come Austria (34.7) and German Federal Republic (31.4), where the rates are high. The lowest in the list is U. S. A. Whites (12.4) followed by New Zealand (14.4, excluding Maoris), Australia (16.4), Canada (16.9) and U. S. A. Nonwhites (17.5). U. S. A. shows low rates, both among the white and the nonwhite males. The rate of Japan here is just about four times that of U. S. A. Whites, an imposing difference indeed.

Among the female population, the highest rate is found in Chile (39.4), followed by Finland (32.3), Japan (30.4), Austria (26.0) and German Federal Republic (24.5). Here, the rate of the Japanese women ranks the third of the 25 countries, and it must be noted that the death rate for stomach cancer is very high in Japan, both among men and women. The lowest rate is shown by the U. S. A. white females (7.2) as was in the case of male population, and next come the U. S. A. nonwhite females (9.6), Australia (11.1) and so forth, the rates being found low in countries outside Europe. The rate here of the U. S. A. Whites is only about onefifth of that of Chile.

4. Comparison of Standardised Death Rates for Cancer of Esophagus

As shown in Table, among males, the death rate for cancer of esophagus is the highest in Switzerland (9.5), France (7.6) being the runner-up, and followed by Finland (5.9), Chile (5.5), Japan (4.9) and U. S. A. Nonwhites (4.7). Here, Japan occupies the 5 th place in the list, and of course belongs to the group of high-rated countries. At the tailend of the list stand Sweden (1.8), Canada (2.0), Norway (2.1) and U. S. A. Whites (2.4). Here, the Whites and Nonwhites in U. S. A. show a considerably perceptible difference in their death rates. The difference

by countries is also rather wide, the highest rate of Switzerland being about five times that of Sweden, the lowest.

Among the females, Finland (5.2) has the highest rate to show, a rate notably higher than the next shown by Chile (3.3). Then come Scotland (3.0), Ireland (2.8), Israel (2.6), Northern Ireland (2.5) and Japan (1.9). The lowest rate is that of the U. S. A. white women (0.7), Union of South Africa (0.8, Europeans only), France (0.9) and Norway (0.9) also belong to the lowest group.

Here, it is worthy of notice that the rates are little different by men and women in Finland. Since, however, our standardising calculation has been made with different standard population for males and females respectively, it may not be fair, strictly speaking, to make direct comparison of the rates for men and women. Another point to be noted here is that France, which belongs to the group of high-rated countries when the male population is considered, has only a low rate for the female population to show.

5. Comparison of Standardised Death Rates for Cancer of Lung

The items "162" and "163" of the detailed list number of the International Lists of Diseases, Injuries and Causes of Death (Sixth Revision) are entitled "Malignant neoplasm of trachea, and of bronchus and lung specified as primary" and "Malignant neoplasm of lung and bronchus, unspecified as to whether primary or secondary". The sums of the rates cited under these two items were used in our standardising calculation. The results are as shown in Table.

Among the male population, Scotland (37.7), England and Wales (35.7), Finland (32.8) and Austria (29.0) have particularly high rates, followed by Netherlands (21.2), Belgium (19.8) and Northern Ireland (19.0). Here, Japan (3.1) is the last in the list, and above her are Norway (6.1), Sweden (6.9), Chile (8.0) and Italy (8.9). This low rate shown by Japan is slightly above one-twelfth that of Scotland at the top in the list.

Among the females, too, Scotland (6.2) tops the list. The runner-up is Israel (5.3), then come England and Wales (5.2), Union of South Africa (4.7) and Austria (4.4). Here too, Japan (1.3) occupies the tailend, and Norway (2.1), Italy (2.4), New Zealand (2.4) and Netherlands (2.5) belong to the countries of low rates.

Here we are struck by the high ranking of the Israel women while the male Israelites stand in the 18th position of the list, while Netherlands shows a high rate among men and a low rate among women. Generally speaking, however, the countries showing high male rates tend to show high rates among females too.

6. Comparison of Standardised Death Rates for Cancer of Breast

As shown in Table, the highest rate of deaths by cancer of breast among women is found in Denmark (20.2), followed by England and Wales (19.5), Scotland

(18.8) and so forth, showing only small differences. Japan is the lowest in the list, with her 3.3, a rate somewhat less than one-sixth of that of Denmark. As the countries with the lowest rates next to Japan may be numbered Chile (6.4), Finland (9.1), Italy (11.1) and France (11.4). Excepting a few countries in the lowest end of the list, the difference in the rates is smaller than in the rates of cancers of other sites, for example, the rate of Italy, the fourth from the tailend of the list, is more than half of that of Denmark, the highest.

7. Comparison of Standardised Death Rates for Cancer of Female Genital Organs

Table. Age-adjusted Death Rates for Malignant Neoplasms by Site in 24 Countries, and their Rank Numbers in Descending Order, 1952-1954

(Rates per 100,000 population, using as standard population that of Japan in 1953. The italic figures show rank numbers. The figures marked by ( ) show the sixth revised international detailed list numbers.)

Country	All sites (140-205)		Stomach (151)		Esophagus (150)	
	Male	Female	Male	Female	Male	Female
Union of South Africa 1) a)	106.2 *7*	103.5 *9*	25.3 *10*	16.5 *13*	2.9 *11*	9.8 *22*
Canada	92.6 *16*	97.8 *13*	16.9 *21*	10.9 *21*	2.0 *23*	1.1 *17*
Chile 3)	97.0 *14*	112.4 *2*	48.1 *2*	39.4 *1*	5.5 *4*	3.3 *2*
U.S.A., White 1)	95.7 *15*	96.7 *15*	12.4 *24*	7.2 *4*	2.4 *21*	0.7 *24*
U.S.A., Nonwhite 1)	97.8 *13*	105.0 *7*	17.5 *20*	9.6 *23*	4.7 *6*	1.1 *15*
Israel b)	83.3 *24*	103.1 *10*	18.4 *19*	15.5 *27*	2.7 *17*	2.6 *5*
Japan	88.1 *21*	77.9 *24*	50.1 *1*	30.4 *13*	4.9 *5*	1.9 *7*
Oerman Federal Republic 2)	106.2 *8*	109.4 *4*	31.4 *5*	24.5 *5*	2.8 *14*	1.2 *14*
Austria	124.6 *3*	111.1 *3*	34.7 *4*	26.0 *4*	3.6 *8*	0.9 *19*
Belgium 4)	106.6 *6*	103.0 *11*	22.9 *11*	16.9 *2*	2.9 *12*	1.5 *10*
Denmark	103.7 *9*	119.1 *1*	21.7 *13*	17.9 *10*	2.5 *19*	1.3 *12*
Finland	134.4 *1*	101.5 *12*	46.4 *3*	32.3 *12*	5.9 *3*	5.2 *1*
France	103.7 *10*	87.6 *21*	19.3 *18*	12.4 *19*	7.6 *2*	0.9 *21*
Ireland	92.2 *18*	90.9 *20*	20.5 *15*	15.9 *15*	2.9 *10*	2.8 *4*
Italy 1)	87.5 *22*	80.7 *23*	27.3 *8*	18.7 *9*	2.8 *13*	0.7 *23*
Norway	88.6 *20*	93.5 *18*	28.7 *6*	19.2 *8*	2.1 *22*	0.9 *20*
Netherlands	103.1 *11*	106.7 *6*	25.3 *9*	19.9 *7*	2.4 *20*	1.3 *13*
Portugal	58.9 *25*	58.0 *25*	.....	.....	.....	.....
England and Wales	120.8 *4*	97.6 *14*	20.1 *17*	13.2 *18*	3.2 *9*	1.9 *8*
Scotland	130.0 *2*	107.3 *5*	22.0 *12*	16.4 *14*	3.9 *7*	3.0 *3*
Northern Ireland	99.6 *12*	95.8 *16*	20.3 *16*	15.7 *16*	2.8 *15*	2.5 *6*
Sweden 1)	83.4 *23*	94.7 *17*	21.6 *14*	17.6 *11*	1.8 *24*	1.1 *16*
Switzerland 1)	120.7 *5*	104.9 *8*	27.9 *7*	22.8 *6*	9.5 *1*	1.4 *11*
Australia	89.5 *19*	86.7 *22*	16.4 *22*	11.1 *20*	2.5 *18*	1.0 *18*
New Zealand c)	92.4 *17*	92.8 *19*	14.4 *23*	10.5 *22*	2.7 *16*	1.8 *9*

Country	Trachea, bronchus and lung (primary and unspecified) (162, 163)		Breast (170)	Uterus (171–174)	Ovary, fallopian tube and broad ligament (175)	Prostate (177)
	Male	Female	Female	Female	Female	Male
Union of South Africa 1) a)	17.2 *10*	4.7 *4*	17.5 *9*	14.6 *6*	5.4 *11*	8.6 *3*
Canada	13.3 *16*	2.8 *15*	19.0 *4*	12.2 *10*	5.5 *8*	7.4 *9*
Chile 3)	8.0 *20*	3.9 *7*	6.4 *23*	19.7 *2*	.....	4.8 *20*
U. S. A., White 1)	15.9 *12*	3.3 *10*	18.0 *8*	12.8 *7*	5.9 *7*	8.1 *7*
U. S. A., Nonwhite 1)	13.6 *14*	3.1 *12*	15.5 *14*	29.2 *1*	4.1 *13*	12.3 *1*
Israel b)	10.2 *18*	5.3 *2*	17.3 *10*	7.0 *22*	.....	4.8 *21*
Japan	3.1 *23*	1.3 *23*	3.3 *24*	17.7 *4*	0.9 *19*	0.5 *24*
German Federal Republic 2)	17.4 *9*	3.2 *11*	12.8 *18*	19.6 *8*	.....	6.3 *16*
Austria	29.0 *4*	4.4 *5*	11.8 *19*	18.5 *3*	7.8 *2*	6.4 *15*
Belgium 4)	19.8 *6*	2.9 *14*	16.4 *12*	12.5 *9*	3.6 *16*	7.5 *8*
Denmark	13.9 *13*	3.4 *9*	20.2 *1*	15.2 *5*	8.5 *1*	7.4 *10*
Finland	32.8 *3*	3.5 *8*	9.1 *22*	11.3 *12*	3.7 *15*	5.8 *19*
France	10.8 *17*	2.7 *16*	11.4 *20*	10.7 *14*	.....	6.1 *17*
Ireland	.....	.....	15.9 *13*	.....	3.5 *17*	3.2 *23*
Italy 1)	8.9 *19*	2.4 *21*	11.1 *21*	11.8 *11*	1.6 *18*	3.7 *22*
Norway	6.1 *22*	2.1 *22*	14.1 *17*	9.1 *21*	7.0 *3*	8.3 *6*
Netherlands	21.2 *5*	2.5 *19*	19.3 *3*	9.2 *20*	6.1 *6*	7.0 *12*
Portugal	.....	.....	.....	.....	.....	.....
England and Wales	35.7 *2*	5.2 *3*	19.5 *2*	9.4 *18*	6.4 *5*	6.7 *18*
Scotland	37.7 *1*	6.2 *1*	18.8 *5*	11.1 *13*	5.5 *9*	6.5 *14*
Northern Ireland	19.0 *7*	4.2 *6*	15.2 *15*	10.0 *16*	3.9 *14*	6.0 *18*
Sweden 1)	6.9 *21*	3.1 *13*	14.7 *16*	.....	.....	7.3 *11*
Switzerland 1)	17.8 *8*	2.6 *18*	18.0 *7*	10.6 *15*	6.9 *4*	8.6 *2*
Australia	13.4 *15*	2.7 *17*	17.1 *11*	9.5 *17*	5.0 *12*	8.5 *4*
New Zealand c)	16.4 *11*	2.4 *20*	18.4 *6*	9.3 *19*	5.4 *10*	8.4 *5*

Note 1) 1951–53 2) 1952, 53 3) 1953 4) 1954
a) European population b) Jewish population c) Excluding Maoris
.....Data not available

A. Uterine Cancer

We have calculated the standardised death rates for uterine cancer from the sums of the deaths listed in compliance with the items 171 : Malignant neoplasm of cervix uteri, 172 : Malignant neoplasm of corpus uteri, 173 : Malignant neoplasm of other parts of uterus including chorionepithelioma and 174 : Malignant neoplasm of uterus unspecified, of the detailed list of the International Lists of Diseases, Injuries and Causes of Death, for use as comparison of death rates for uterine cancer. It is of course preferable to make such studies about uterine cervix cancer and uterine corpus cancer as separate items, but in many countries, including Japan, the detailed part of uterus is apt to be not mentioned on the death

certificates, so that we cannot make comparisons by cervix and corpus separaiely.

The death rates for chorionepithelioma is included in the above total rates, but since no country except Japan has a separate item for it, the rates cannot be counted out in our studies. The number of deaths by chorionepithelioma, however, was only 258 in the whole year of 1954 in Japan, and as the rates in the other countries are also inferred to be lower than in Japan, the portion due to this disease in the total death rate for uterine cancer is probably extremely small anyhow.

The standardised death rates for uterine cancer, as shown in Table, is the highest in U. S. A. Nonwhites (29.2), next in Chile (19.7), Austria (18.5), Japan (17.7) and Denmark (15.2). The countries showing low rates are Israel (7.0), Norway (9.1), Netherlands (9.2), New Zealand (9.3) and England and Wales (9.4). Israel is known for her low frequency of cancer of cervix uteri, so the above rate is apparently due to corpus cancer in the largest portion. Japan is the fourth country of high death rate for uterine cancer among the 22 countries cited.

B. Cancer of Ovary, Fallopian Tube and Broad Ligament

This item stands in No. 175 of the international detailed list. In the Japanese statistics, the deaths due to ovarian cancer are separately listed under the subitem "175. a", but no other country has yet adopted the subclassification. Since, however, in Japan, the deaths falling under the item "175" counted only 455, of which only 6 died of of cancer outside the ovaries, we can infer that the deaths listed under "175" consist nearly entirely of ovarian cancer cases, though we have no adequate information in this respect in the other countries cited.

The standardised death rates for the cancer of these sites have been computed for 19 countries, as shown in Table. The highest rate here is shown by Denmark (8.5), Austria (7.8), Norway (7.0), Switzerland (6.9) and England and Wales (6.4) in the order named. Japan (0.9), Italy (1.6), Ireland (3.5), Belgium (3.6) and Finland (3.7) stand low in the list. The lowest rate, Japan's one, is less than one-ninth of the highest, Denmark's rate. It is noteworthy that in the rates for cancer both of the breast and the ovary, Denmark and Japan occupy the top and the Tailend respectively.

8. Comparison of the Standardised Death Rates for Cancer of Prostate

As shown in Table, the rate is highest among the U. S. A. Nonwhites (12.3) and rather conspicuously so. Next comes Switzerland (8.6), followed in descending order by the Union of South Africa (8.6), Australia (8.5), New Zealand (8.4), Norway (8.3), and the U. S. A. Whites (8.1), all showing rates above 8. The rate is exceptionally low in Japan (0.5), Ireland (3.2), Itary (3.7), Israel (4.8) and Chile (4.8) belonging to the countries of low rates. It is to be noted that the death rate for this disease in Japan amounts only to about one-twentyfifth of the highest

rate among the U. S. A. Nonwhites.

Summary

The authors have computed the standardised death rates of some selected countries, with the relevant data for 1951-1954 kindly furnished us by the central statistics offices of the countries as basis and with the sex and age distribution of the Japanese population as standard, and using these standardised death rates, made a geographical observation on the mortality from cancer of all sites, and of stomach, esophagus, lung, breast, uterus, ovary, and prostate.

References

1) Data furnished upon application by the central offices of vital statistics of 23 countries.
2) Welfare Ministry: Vital Statistics, Japan, 1952, 1953 and 1954

要　旨

訂正死亡率による諸国癌死亡の地理的観察

瀬木三雄，藤咲遖，栗原　登
(東北大学医学部公衆衛生学教室)

著者等は 23 カ国 (南阿連邦，カナダ，チリー，米国，イスラエル，西ドイツ，オーストリア，ベルギー，デンマーク，フィンランド，フランス，アイルランド，イタリー，ノールエー，オランダ，ポルトガル，英国，スコットランド，北アイルランド，スエーデン，スイス，オーストラリア，ニュージーランド) の中央統計官庁より直接入手せる 1952-54 年の癌死亡資料により，1953 年の日本の性別人口を標準とする年令訂正死亡率を，全部位の癌並びに胃，食道，肺，乳房，子宮，卵巣及び前立腺の各部位の癌について算出し，これを比較し，諸国におけるその率を順位づけた。

SUPPLEMENT 1 TO THE ATLAS OF EXFOLIATIVE CYTOLOGY

G.N. Papanicolaou, Harvard University Press, Cambridge, Mass., 1956. The author has had primarily an intention to issue supplemental leaves for the Atlas of Exfoliative Cytology to keep his book and knowledge of the readers abreast with the advances in the field of exfoliative cytology. The first supplement was made available last summer to be incorporated in the original book which appeared in loose-leaf form. Sixteen plates, all reproduction of color photographs, and brief discussion on each material were added this time. The majority of the new illustrations are to serve correlating cytologic and histopathologic findings. Smears and sections of the materials from the female-genital, urinary, male-genital, respiratory and digestive systems, as well as from the breast and exudates are presented in pictures and discussed. In contrast to the artist's paintings which featured the original part, the color photographs appear less luminous, but much nearer to the actual impressions on materials which the readers are expected to deal with in their routine works. Examples of diagnosis of origin of some secondary cancers and differentiation of such metastatic tumors from the primary neoplasms of certain organs are also presented, which is not always possible by the cytologic technique only. In the female genital system, a few leaves are dedicate to the carcinoma in situ of the uterine cervix. The entire presentation is much the same as the previously published part and very helpful to all the specialists and to others who may eventually become interested in this line of study. (K. Oota)

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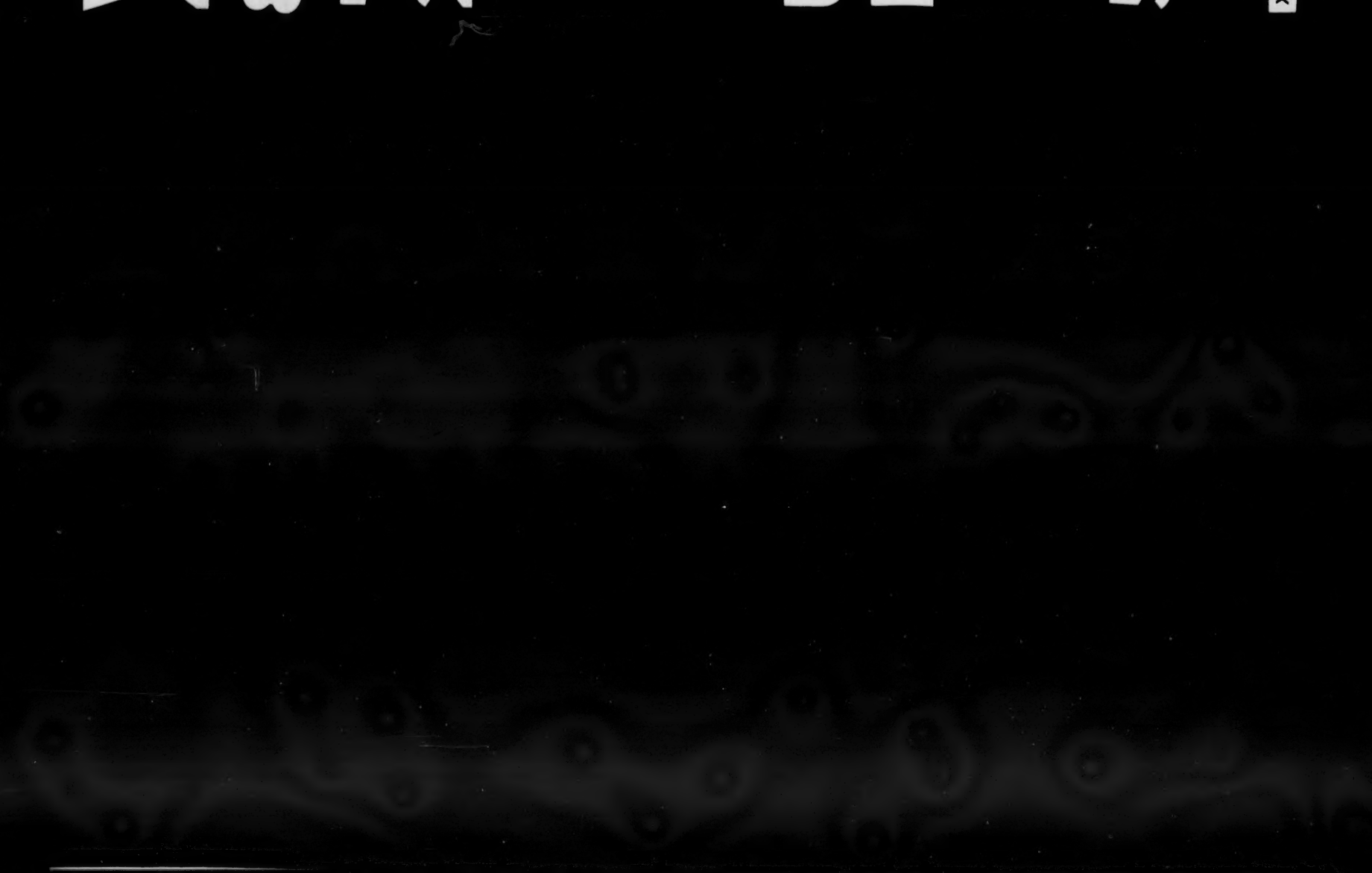
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